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APPLICATION NUMBER: *60/537,341*
FILING DATE: *January 16, 2004*
RELATED PCT APPLICATION NUMBER: *PCT/US05/01469*

Certified by

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BAKER BOTTS L.L.P

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Attorney Docket No. P36134
Express Mail Label No. ER589237548US

U.S. PTO 60/537341 01/16/04

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Margarita	Garcia-Calvo	Westfield, New Jersey

☐ *Additional inventors are being named on the ___ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (280 characters max)

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification *Number of Pages* 203
☑ Drawing(s) *Number of Sheets* 11
☐ Application Data Sheet. See 37 CFR 1.76
☐ CD(s), Number
☐ Other (specify)

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☐ Applicant claims small entity status. See 37 CFR 1.27.
☑ A check or money order is enclosed to cover the filing fees
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FILING FEE AMOUNT ($): 160

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☑ No.
☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE Rochelle K. Seide

Date: Jan. 16, 2004

TYPED or PRINTED NAME Rochelle K. Seide

REGISTRATION NO. *(if appropriate)* 32,300

Docket Number: P36134

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USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

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FEE TRANSMITTAL for FY 2003

Effective 10/01/2003. Patent fees are subject to annual revision.

☐ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 160

Complete if Known	
Application Number	To Be Assigned
Filing Date	January 16, 2004
First Named Inventor	Garcia-Calvo
Examiner Name	N/A
Art Unit	N/A
Attorney Docket No.	P36134

METHOD OF PAYMENT *(check all that apply)*

☑ Check ☐ Credit card ☐ Money Order ☐ Other ☐ None

☑ Deposit Account:

Deposit Account Number: 02-4377

Deposit Account Name: Baker Botts LLP

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FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1001	770	2001	385	Utility filing fee	
1002	340	2002	170	Design filing fee	
1003	530	2003	265	Plant filing fee	
1004	770	2004	385	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	160

SUBTOTAL (1) ($) 160

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

		Extra Claims		Fee from below	Fee Paid
Total Claims	- 20 =	0	X		0
Independent Claims	- 3 =	0	X		0
Multiple Dependent					

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	86	2201	43	Independent claims in excess of 3
1203	290	2203	145	Multiple dependent claim, if not paid
1204	86	2204	43	** Reissue independent claims over original patent
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($) 0

***or number previously paid, if greater; For Reissues, see above*

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	420	2252	210	Extension for reply within second month	
1253	950	2253	475	Extension for reply within third month	
1254	1,480	2254	740	Extension for reply within fourth month	
1255	2,010	2255	1,005	Extension for reply within fifth month	
1401	330	2401	165	Notice of Appeal	
1402	330	2402	165	Filing a brief in support of an appeal	
1403	290	2403	145	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive - unavoidable	
1453	1,300	2453	650	Petition to revive - unintentional	
1501	1,330	2501	665	Utility issue fee (or reissue)	
1502	480	2502	240	Design issue fee	
1503	630	2503	315	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	770	2809	385	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	770	2810	385	For each additional invention to be examined (37 CFR 1.129(b))	
1801	770	2801	385	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify) _____

*Reduced by Basic Filing Fee Paid

SUBTOTAL (3) ($) 0

SUBMITTED BY				(Complete *if applicable*)	
Name (Print/Type)	Rochelle K. Seide	*Registration No. (Attorney/Agent)*	32,300	*Telephone*	212-408-2500
Signature	Rochelle K. Seide			*Date*	Jan. 16, 2004

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BAKER BOTTS L.L.P.

30 ROCKEFELLER PLAZA

NEW YORK, NEW YORK 10112

TO ALL WHOM IT MAY CONCERN:

Be it known that I, Margarita GARCIA-CALVO, have invented an improvement in

NPC1L1 (NPC3) AND METHODS OF IDENTIFYING LIGANDS THEREOF

of which the following is a

SPECIFICATION

FIELD OF THE INVENTION

[0001] The present invention includes NPC1L1 polypeptides and polynucleotides which encode the polypeptides, methods of use and methods of identifying modulators and ligands thereof.

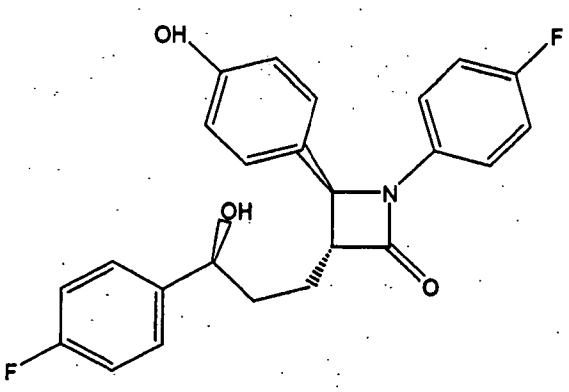
BACKGROUND OF THE INVENTION

[0002] A factor leading to development of vascular disease, a leading cause of death in industrialized nations, is elevated serum cholesterol. It is estimated that 19% of Americans between the ages of 20 and 74 years of age have high serum cholesterol. The most prevalent form of vascular disease is arteriosclerosis, a condition associated with the thickening and hardening of the arterial wall. Arteriosclerosis of the large vessels is referred to as atherosclerosis. Atherosclerosis is the predominant underlying factor in vascular disorders such

as coronary artery disease, aortic aneurysm, arterial disease of the lower extremities and cerebrovascular disease.

[0003] Cholesteryl esters are a major component of atherosclerotic lesions and the major storage form of cholesterol in arterial wall cells. Formation of cholesteryl esters is also a step in the intestinal absorption of dietary cholesterol. Thus, inhibition of cholesteryl ester formation and reduction of serum cholesterol can inhibit the progression of atherosclerotic lesion formation, decrease the accumulation of cholesteryl esters in the arterial wall, and block the intestinal absorption of dietary cholesterol.

[0004] The regulation of whole-body cholesterol homeostasis in mammals and animals involves the regulation of intestinal cholesterol absorption, cellular cholesterol trafficking, dietary cholesterol and modulation of cholesterol biosynthesis, bile acid biosynthesis, steroid biosynthesis and the catabolism of the cholesterol-containing plasma lipoproteins. Regulation of intestinal cholesterol absorption has proven to be an effective means by which to regulate serum cholesterol levels. For example, a cholesterol absorption inhibitor, ezetimibe (



), has been shown to be effective in this regard. A pharmaceutical composition containing ezetimibe is commercially available from Merck/Schering-Plough Pharmaceuticals, Inc. under

the tradename Zetia®. Identification of a gene target through which ezetimibe acts is important to understanding the process of cholesterol absorption and to the development of other, novel absorption inhibitors. The present invention addresses this need by providing a rat and a mouse homologue of human NPC1L1 (also known as NPC3; Genbank Accession No. AF192522; Davies, *et al.*, (2000) Genomics 65(2): 137-45 and Ioannou, (2000) Mol. Genet. Metab.71(1-2): 175-81), an ezetimibe target.

[0005] NPC1L1 is an N-glycosylated protein comprising a YQRL (SEQ ID NO: 38) motif (i.e., a *trans*-golgi network to plasma membrane transport signal; see Bos, *et al.*, (1993) EMBO J. 12: 2219-2228; Humphrey, *et al.*, (1993) J. Cell. Biol. 120: 1123-1135; Ponnambalam, *et al.*, (1994) J. Cell. Biol. 125: 253-268 and Rothman, *et al.*, (1996) Science 272: 227-234) which exhibits limited tissue distribution and gastrointestinal abundance. Also, the human *NPC1L1* promoter includes a Sterol Regulated Element Binding Protein 1 (SREBP1) binding consensus sequence (Athanikar, *et al.*, (1998) Proc. Natl. Acad. Sci. USA 95: 4935-4940; Ericsson, *et al.*, (1996) Proc. Natl. Acad. Sci. USA 93: 945-950; Metherall, *et al.*, (1989) J. Biol. Chem. 264: 15634-15641; Smith, *et al.*, (1990) J. Biol. Chem. 265: 2306-2310; Bennett, *et al.*, (1999) J. Biol. Chem. 274: 13025-13032 and Brown, *et al.*, (1997) Cell 89: 331-340). NPC1L1 has 42% amino acid sequence homology to human NPC1 (Genbank Accession No. AF002020), a receptor responsible for Niemann-Pick C1 disease (Carstea, *et al.*, (1997) Science 277: 228-231). Niemann-Pick C1 disease is a rare genetic disorder in humans which results in accumulation of low density lipoprotein (LDL)-derived unesterified cholesterol in lysosomes (Pentchev, *et al.*, (1994) Biochim. Biophys. Acta. 1225: 235-243 and Vanier, *et al.*, (1991) Biochim. Biophys. Acta. 1096: 328-337). In addition, cholesterol accumulates in the *trans*-golgi network of npc1$^-$

cells, and relocation of cholesterol, to and from the plasma membrane, is delayed. NPC1 and NPC1L1 each possess 13 transmembrane spanning segments as well as a sterol-sensing domain (SSD). Several other proteins, including HMG-CoA Reductase (HMG-R), Patched (PTC) and Sterol Regulatory Element Binding Protein Cleavage-Activation Protein (SCAP), include an SSD which is involved in sensing cholesterol levels possibly by a mechanism which involves direct cholesterol binding (Gil, *et al.*, (1985) Cell 41: 249-258; Kumagai, *et al.*, (1995) J. Biol. Chem. 270: 19107-19113 and Hua, *et al.*, (1996) Cell 87: 415-426).

SUMMARY OF THE INVENTION

[0006] The present invention is based on the discovery that NPC1L1 is the target through which ezetimibe act, and consequently plays a critical role in the regulation of sterol and 5α-stanol intestinal transport and absorption, e.g. cholesterol absorption. Accordingly, this invention provides for the use of NPC1L1 in an assay for identifying ligands that block NPC1L1-mediated sterol and 5α-stanol intestinal transport. The present invention provides methods for identifying ligands of NPC1L1 which involve contacting NPC1L1 with a detectably labeled substituted 2-azetidinone, preferably substituted 2-azetidinone-glucuronide, and a candidate compound, and determining whether the candidate compound binds to NPC1L1. The modulation of the binding of the substituted 2-azetidinone to NPC1L1 by the binding of the candidate compound to NPC1L1 indicates that the candidate compound is a ligand that binds to NPC1L1 and is an inhibitor of sterol and 5α-stanol absorption.

[0007] The present invention also provides a method for identifying a ligand of NPC1L1 comprising contacting NPC1L1 with a detectably labeled substituted 2-azetidinone, preferably substituted 2-azetidinone-glucuronide, and measuring the binding of detectably labeled

substituted 2-azetidinone to NPC1L1 in the presence and absence of a candidate compound, wherein decreased binding of the detectably labeled substituted 2-azetidinone to the NPC1L1 in the presence of the candidate compound indicates that said candidate compound is a ligand of NPC1L1 and is an inhibitor of sterol and 5α-stanol absorption.

[0008] The present invention also provides for a method for identifying a compound that inhibits intestinal sterol or 5α-stanol absorption mediated by NPC1L1 involving contacting NPC1L1 with a detectably labeled ligand and the candidate compound and determining whether the candidate compound binds to NPC1L1, wherein binding of said candidate compound to NPC1L1 modulates binding of said ligand to NPC1L1, wherein said modulation indicates that the candidate compound is an intestinal sterol or 5α-stanol absorption inhibitor.

[0009] The present invention provides methods for identifying an ligand of NPC1L1 comprising (a) contacting a host cell (*e.g.*, chinese hamster ovary (CHO) cell, a J774 cell, a macrophage cell or a Caco2 cell) expressing a polypeptide comprising the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 4 or SEQ ID NO: 12 or a functional fragment thereof on a cell surface, in the presence of a known amount of a detectably labeled (*e.g.*, with ^{3}H, ^{14}C, ^{125}I, ^{35}S or fluorescence labeling) substituted azetidinone (*e.g.*, ezetimibe), with a sample to be tested for the presence of an NPC1L1 ligand; and (b) measuring the amount of detectably labeled substituted azetidinone (*e.g.*, ezetimibe) specifically bound to the polypeptide; wherein an NPC1L1 ligand in the sample is identified by measuring substantially reduced binding of the detectably labeled substituted azetidinone (*e.g.*, ezetimibe) to the polypeptide, compared to what would be measured in the absence of such a ligand.

[0010] Another method for identifying an ligand of NPC1L1 is also provided. The method comprises (a) placing, in an aqueous suspension, a plurality of support particles, impregnated with a fluorescer (*e.g.*, yttrium silicate, yttrium oxide, diphenyloxazole and polyvinyltoluene), to which a host cell (*e.g.*, chinese hamster ovary (CHO) cell, a J774 cell, a macrophage cell or a Caco2 cell) expressing a polypeptide comprising the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 4 or SEQ ID NO: 12 or a functional fragment thereof on a cell surface are attached; (b) adding, to the suspension, a radiolabeled (*e.g.*, with ^{3}H, ^{14}C or ^{125}I) substituted azetidinone (*e.g.*, ezetimibe) and a sample to be tested for the presence of a ligand, wherein the radiolabel emits radiation energy capable of activating the fluorescer upon the binding of the substituted azetidinone (*e.g.*, ezetimibe) to the polypeptide to produce light energy, whereas radiolabeled substituted azetidinone (*e.g.*, ezetimibe) that does not bind to the polypeptide is, generally, too far removed from the support particles to enable the radioactive energy to activate the fluorescer; and (c) measuring the light energy emitted by the fluorescer in the suspension; wherein an NPC1L1 ligand in the sample is identified by measuring substantially reduced light energy emission, compared to what would be measured in the absence of such a ligand.

[0011] Also provided is a method for identifying a ligand of NPC1L1 comprising (a) contacting a host cell (*e.g.*, Chinese hamster ovary (CHO) cell, a J774 cell, a macrophage cell or a Caco2 cell) expressing a polypeptide comprising an amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 4 or SEQ ID NO: 12 or a functional fragment thereof on a cell surface with detectably labeled (*e.g.*, with ^{3}H, ^{14}C or ^{125}I) sterol (*e.g.*, cholesterol) or 5α-stanol and with a sample to be tested for the presence of an ligand; and (b) measuring the amount of detectably labeled sterol (*e.g.*, cholesterol) or 5α-stanol in the cell; wherein an NPC1L1 antagonist in the

sample is identified by measuring substantially reduced detectably labeled sterol (*e.g.*, cholesterol) or 5α-stanol within the host cell, compared to what would be measured in the absence of such an antagonist and wherein an NPC1L1 agonist in the sample is identified by measuring substantially increased detectably labeled sterol (*e.g.*, cholesterol) or 5α-stanol within the host cell, compared to what would be measured in the absence of such an agonist.

[0012] The present invention includes methods for inhibiting NPC1L1-mediated intestinal sterol (*e.g.*, cholesterol) or 5α-stanol uptake, in a subject, by administering a substance identified by the screening methods described herein to the subject. Such substances include compounds such as small molecule antagonists of NPC1L1 other than ezetimibe. Also contemplated are methods for antagonizing NPC1L1-mediated sterol (*e.g.*, cholesterol) or 5α-stanol absorption by administering anti-NPC1L1 antibodies. NPC1L1-mediated absorption of sterol (*e.g.*, cholesterol) or 5α-stanol can also be antagonized by any method which reduces expression of NPC1L1 in an organism. For example, NPC1L1 expression can be reduced by introduction of anti-sense *NPC1L1* mRNA into a cell of an organism or by genetic mutation of the *NPC1L1* gene in an organism (*e.g.*, by complete knockout, disruption, truncation or by introduction of one or more point mutations).

[0013] Also included in the present invention is a mutant transgenic mammal (*e.g.*, mouse, rat, dog, rabbit, pig, guinea pig, cat, horse), preferably a mouse comprising a homozygous or heterozygous mutation (*e.g.*, disruption, truncation, one or more point mutations, knock out) of endogenous, chromosomal *NPC1L1* wherein, preferably, the mouse does not produce any functional NPC1L1 protein. Preferably, the mutant mouse, lacking functional NPC1L1, exhibits

a reduced level of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption and/or a reduced level of serum sterol (*e.g.*, cholesterol) or 5α-stanol and/or a reduced level of liver sterol (*e.g.*, cholesterol) or 5α-stanol as compared to that of a non-mutant mouse comprising functional NPC1L1. Preferably, in the mutant mouse chromosome, the region of *NPC1L1* (SEQ ID NO: 45) deleted is from nucleotide 790 to nucleotide 998. In one embodiment, *NPC1L1* (SEQ ID NO: 11) is deleted from nucleotide 767 to nucleotide 975. Any offspring or progeny of a parent *NPC1L1* mutant mouse (i.e., *npc1l1*) of the invention which has inherited an *npc1l1* mutant allele is also part of the present invention.

[0014] The scope of the present invention also includes a method for screening a sample for an intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption antagonist comprising (a) feeding a sterol (*e.g.*, cholesterol) or 5α-stanol-containing substance (*e.g.*, comprising radiolabeled cholesterol, such as ^{14}C-cholesterol or ^{3}H-cholesterol) to a first and second mouse comprising a functional *NPC1L1* gene and to a third, mutant mouse lacking a functional *NPC1L1*; (b) administering the sample to the first mouse comprising a functional *NPC1L1* but not to the second mouse; (c) measuring the amount of sterol (*e.g.*, cholesterol) or 5α-stanol absorption in the intestine of said first, second and third mouse (*e.g.*, by measuring serum cholesterol); and (d) comparing the levels of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption in each mouse; wherein the sample is determined to contain the intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption antagonist when the level of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption in the first mouse and third mouse are less than the amount of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption in the second mouse.

[0015] The present invention also encompasses a kit comprising (a) a substituted azetidinone (*e.g.*, ezetimibe) in a pharmaceutical dosage form (*e.g.*, a pill or tablet comprising 10 mg substituted azetidinone (*e.g.*, ezetimibe)); and (b) information, for example in the form of an insert, indicating that NPC1L1 is a target of ezetimibe. The kit may also include simvastatin in a pharmaceutical dosage form (*e.g.*, a pill or tablet comprising 5 mg, 10 mg, 20 mg, 40 mg or 80 mg simvastatin). The simvastatin in pharmaceutical dosage form and the ezetimibe in pharmaceutical dosage form can be associated in a single pill or tablet or in separate pills or tablets.

[0016] The present invention also provides any isolated mammalian cell (*e.g.*, isolated mouse cell, isolated rat cell or isolated human cell) which lacks a gene which encodes or can produce a functional NPC1L1 polypeptide. The isolated cell can be isolated from a mutant mouse comprising a homozygous mutation of endogenous, chromosomal *NPC1L1* wherein the mouse does not produce any functional NPC1L1 protein. Further, the mutation can be in a gene which when un-mutated encodes an amino acid sequence of SEQ ID NO: 12 (*e.g.*, comprising a nucleotide sequence of SEQ ID NO: 11). The cell can be isolated or derived from duodenum, gall bladder, liver, small intestine or stomach tissue. The cell can be an enterocyte.

BRIEF DESCRIPTION OF THE FIGURES

[0017] Figure 1A shows an equilibrium saturation binding plot exhibiting the binding of ^{3}H-EZE-glucuronide to rhesus brush border membrane vesicles.

[0018] Figure 1B shows a scatchard analysis of ^{3}H-EZE-glucuronide binding to rhesus brush border membrane vesicles.

[0019] Figure 2A shows an equilibrium saturation binding plot exhibiting the binding of ^{3}H-EZE-glucuronide to rat brush border membrane vesicles.

[0020] Figure 2B shows a scatchard analysis of ^{3}H-EZE-glucuronide binding to rat brush border membrane vesicles.

[0021] Figure 3A shows association kinetic analysis of ^{3}H-EZE-glucuronide in rat brush border membrane vesicles.

[0022] Figure 3B shows dissociation kinetic analysis of ^{3}H-EZE-glucuronide in rat brush border membrane vesicles.

[0023] Figure 4A shows association kinetic analysis of ^{3}H-EZE-glucuronide in rhesus brush border membrane vesicles.

[0024] Figure 4B shows dissociation kinetic analysis of ^{3}H-EZE-glucuronide in rhesus brush border membrane vesicles.

[0025] Figure 5 shows the results of a binding assay where ^{3}H-EZE-glucuronide is dissociated by EZE-glucuronide and compound **2** from rhesus (A) and rat (B) brush border membrane vesicles.

[0026] Figure 6 shows the results of a binding assay where ^{35}S-**2** is dissociated by EZE-glucuronide and **2** from mouse brush border membrane vesicles.

[0027] Figure 7 shows the distribution of ^{3}H-EZE-glucuronide binding to various portions of rhesus (A) and rat (B) intestinal tissue.

[0028] Figure 8 shows the results of a binding assay where ^{35}S-**2** is dissociated by EZE-glucuronide and various analogs from CHO cells transfected with rat NPC1L1.

[0029] Figure 9 shows the results of a binding assay where ^{35}S-**2** is dissociated by EZE-glucuronide and various analogs from CHO cells transfected with human NPC1L1.

[0030] Figure 10 shows the binding of ^{35}S-**2** to brush border membrane vesicles prepared from wild type (A) and *NPC1L1* knockout (-/-) mice.

[0031] Figure 11 shows the results of a binding assay where ^{35}S-**2** is dissociated by compound **2** from mouse wild type (A) and *NPC1L1* knockout (-/-) (B) brush border membrane vesicles.

DETAILED DESCRIPTION OF THE INVENTION

[0032] The present invention includes an NPC1L1 polypeptide from rat, human and from mouse along with polynucleotides encoding the respective polypeptides. Preferably, the rat NPC1L1 polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 2, the human NPC1L1 comprises the amino acid sequence set forth in SEQ ID NO: 4 and the mouse NPC1L1 polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 12. The rat *NPC1L1* polynucleotide of SEQ ID NO: 1 or 10 encodes the rat NPC1L1 polypeptide. The human *NPC1L1* polynucleotide of SEQ ID NO: 3 encodes the human NPC1L1 polypeptide. The mouse *NPC1L1* polynucleotide of SEQ ID NO: 11 or 13 encodes the mouse NPC1L1 polypeptide.

[0033] The present invention includes any isolated polynucleotide or isolated polypeptide comprising a nucleotide or amino acid sequence referred to, below, in Table 1.

Table 1. Polynucleotides and Polypeptides of the Invention.

Polynucleotide or Polypeptide	Sequence Identifier
Rat *NPC1L1* polynucleotide	SEQ ID NO: 1
Rat *NPC1L1* polypeptide	SEQ ID NO: 2
Human *NPC1L1* polynucleotide	SEQ ID NO: 3
Human *NPC1L1* polypeptide	SEQ ID NO: 4
Rat *NPC1L1* expressed sequence tag 603662080F1 (partial sequence)	SEQ ID NO: 5
Rat *NPC1L1* expressed sequence tag 603665037F1 (partial sequence)	SEQ ID NO: 6
Rat *NPC1L1* expressed sequence tag 604034587F1 (partial sequence)	SEQ ID NO: 7
EST 603662080F1 with downstream sequences added	SEQ ID NO: 8
EST 603662080F1 with upstream and downstream sequences added	SEQ ID NO: 9
Back-translated polynucleotide sequence of rat NPC1L1	SEQ ID NO: 10
Mouse NPC1L1 polynucleotide	SEQ ID NO: 11
Mouse NPC1L1 polypeptide	SEQ ID NO: 12
Back-translated polynucleotide sequence of mouse NPC1L1	SEQ ID NO: 13
Back-translated polynucleotide sequence of human NPC1L1	SEQ ID NO: 51

[0034] A human NPC1L1 is also disclosed under Genbank Accession Number AF192522. As discussed below, the nucleotide sequence of the rat *NPC1L1* set forth in SEQ ID NO: 1 was obtained from an expressed sequence tag (EST) from a rat jejunum enterocyte cDNA library.

SEQ ID NOs: 5-7 include partial nucleotide sequences of three independent cDNA clones. The downstream sequence of the SEQ ID NO: 5 EST (603662080F1) were determined; the sequencing data from these experiments are set forth in SEQ ID NO: 8. The upstream sequences were also determined; these data are set forth in SEQ ID NO: 9.

[0035] SEQ ID NOs: 43 and 44 are the nucleotide and amino acid sequence, respectively, of human NPC1L1 which is disclosed under Genbank Accession No.: AF192522 (see Davies, *et al.*, (2000) Genomics 65(2): 137-45).

[0036] SEQ ID NO: 45 is the nucleotide sequence of a mouse *NPC1L1* which is disclosed under Genbank Accession No. AK078947.

[0037] NPC1L1 mediates intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption. Inhibition of NPC1L1 in a patient is a useful method for reducing intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption and serum sterol (*e.g.*, cholesterol) or 5α-stanol in the patient. Reducing the level of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption and serum sterol (*e.g.*, cholesterol) or 5α-stanol in a patient is a useful way in which to treat or prevent the occurrence of atherosclerosis, particularly diet-induced atherosclerosis.

[0038] As used herein, the term "sterol" includes, but is not limited to, cholesterol and phytosterols (including, but not limited to, sitosterol, campesterol, stigmasterol and avenosterol).

[0039] As used herein, the term "5α-stanol" includes, but is not limited to, cholestanol, 5α-campestanol and 5α-sitostanol.

Molecular Biology

[0040] In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook, *et al.*, 1989"); DNA Cloning: A Practical Approach, Volumes I and II (D.N. Glover ed. 1985); Oligonucleotide Synthesis (M.J. Gait, ed. 1984); Nucleic Acid Hybridization (B.D. Hames & S.J. Higgins, eds. (1985)); Transcription And Translation (B.D. Hames & S.J. Higgins, eds. (1984)); Animal Cell Culture (R.I. Freshney, ed. (1986)); Immobilized Cells And Enzymes (IRL Press, (1986)); B. Perbal, A Practical Guide To Molecular Cloning (1984); F.M. Ausubel, *et al.* (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (1994).

[0041] The back-translated sequences of SEQ ID NO: 10 and of SEQ ID NO: 13 uses the single-letter code shown in Table 1 of Annex C, Appendix 2 of the PCT Administrative Instruction in the Manual of Patent Examination Procedure.

[0042] A "polynucleotide", "nucleic acid " or "nucleic acid molecule" may refer to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"), or any phosphoester analogs thereof, such as phosphorothioates and thioesters, in single stranded form, double-stranded form or otherwise.

[0043] A "polynucleotide sequence", "nucleic acid sequence" or "nucleotide sequence" is a series of nucleotide bases (also called "nucleotides") in a nucleic acid, such as DNA or RNA, and means any chain of two or more nucleotides.

[0044] A "coding sequence" or a sequence "encoding" an expression product, such as a RNA, polypeptide, protein, or enzyme, is a nucleotide sequence that, when expressed, results in production of the product.

[0045] The term "gene" means a DNA sequence that codes for or corresponds to a particular sequence of ribonucleotides or amino acids which comprise all or part of one or more RNA molecules, proteins or enzymes, and may or may not include regulatory DNA sequences, such as promoter sequences, which determine, for example, the conditions under which the gene is expressed. Genes may be transcribed from DNA to RNA which may or may not be translated into an amino acid sequence.

[0046] The present invention includes nucleic acid fragments of any of SEQ ID NOs: 1, 5-11 or 13. A nucleic acid "fragment" includes at least about 30 (*e.g.*, 31, 32, 33, 34), preferably at least about 35 (e.g, 25, 26, 27, 28, 29, 30, 31, 32, 33 or 34), more preferably at least about 45 (*e.g.*, 35, 36, 37, 38, 39, 40, 41, 42, 43 or 44), and most preferably at least about 126 or more contiguous nucleotides (*e.g.*, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 150, 160, 170, 180, 190, 200, 300, 400, 500, 1000 or 1200) from any of SEQ ID NOs: 1, 5-11 or 13.

[0047] The present invention also includes nucleic acid fragments consisting of at least about 7 (*e.g.*, 9, 12, 17, 19), preferably at least about 20 (*e.g.*, 30, 40, 50, 60), more preferably about 70 (*e.g.*, 80, 90, 95), yet more preferably at least about 100 (*e.g.*, 105, 110, 114) and even more

preferably at least about 115 (*e.g.*, 117, 119, 120, 122, 124, 125, 126) contiguous nucleotides from any of SEQ ID NOs: 1, 5-11 or 13.

[0048] As used herein, the term "oligonucleotide" refers to a nucleic acid, generally of no more than about 100 nucleotides (*e.g.*, 30, 40, 50, 60, 70, 80, or 90), that may be hybridizable to a genomic DNA molecule, a cDNA molecule, or an mRNA molecule encoding a gene, mRNA, cDNA, or other nucleic acid of interest. Oligonucleotides can be labeled, *e.g.*, by incorporation of ^{32}P-nucleotides, ^{3}H-nucleotides, ^{14}C-nucleotides, ^{35}S-nucleotides or nucleotides to which a label, such as biotin, has been covalently conjugated. In one embodiment, a labeled oligonucleotide can be used as a probe to detect the presence of a nucleic acid. In another embodiment, oligonucleotides (one or both of which may be labeled) can be used as PCR primers, either for cloning full length or a fragment of the gene, or to detect the presence of nucleic acids. Generally, oligonucleotides are prepared synthetically, preferably on a nucleic acid synthesizer.

[0049] A "protein sequence", "peptide sequence" or "polypeptide sequence" or "amino acid sequence" may refer to a series of two or more amino acids in a protein, peptide or polypeptide.

[0050] "Protein", "peptide" or "polypeptide" includes a contiguous string of two or more amino acids. Preferred peptides of the invention include those set forth in any of SEQ ID NOs: 2 or 12 as well as variants and fragments thereof. Such fragments preferably comprise at least about 10 (*e.g.*, 11, 12, 13, 14, 15, 16, 17, 18 or 19), more preferably at least about 20 (*e.g.*, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40), and yet more preferably at least about 42 (*e.g.*, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, 110, 120 or 130) or more contiguous amino acid residues from any of SEQ ID NOs: 2 or 12.

[0051] The present invention also includes polypeptides, preferably antigenic polypeptides, consisting of at least about 7 (*e.g.*, 9, 10, 13, 15, 17, 19), preferably at least about 20 (*e.g.*, 22, 24, 26, 28), yet more preferably at least about 30 (*e.g.*, 32, 34, 36, 38) and even more preferably at least about 40 (*e.g.*, 41, 42) contiguous amino acids from any of SEQ ID NOs: 2 or 12.

[0052] The polypeptides of the invention can be produced by proteolytic cleavage of an intact peptide, by chemical synthesis or by the application of recombinant DNA technology and are not limited to polypeptides delineated by proteolytic cleavage sites. The polypeptides, either alone or cross-linked or conjugated to a carrier molecule to render them more immunogenic, are useful as antigens to elicit the production of antibodies and fragments thereof. The antibodies can be used, *e.g.*, in immunoassays for immunoaffinity purification or for inhibition of NPC1L1, etc.

[0053] The terms "isolated polynucleotide" or "isolated polypeptide" include a polynucleotide (*e.g.*, RNA or DNA molecule, or a mixed polymer) or a polypeptide, respectively, which are partially or fully separated from other components that are normally found in cells or in recombinant DNA expression systems. These components include, but are not limited to, cell membranes, cell walls, ribosomes, polymerases, serum components and extraneous genomic sequences.

[0054] An isolated polynucleotide or polypeptide will, preferably, be an essentially homogeneous composition of molecules but may contain some heterogeneity.

[0055] "Amplification" of DNA as used herein may denote the use of polymerase chain reaction (PCR) to increase the concentration of a particular DNA sequence within a mixture of DNA sequences. For a description of PCR see Saiki, *et al.*, Science (1988) 239: 487.

[0056] The term "host cell" includes any cell of any organism that is selected, modified, transfected, transformed, grown, or used or manipulated in any way, for the production of a substance by the cell, for example, the expression or replication, by the cell, of a gene, a DNA or RNA sequence or a protein. Preferred host cells include chinese hamster ovary (CHO) cells, murine macrophage J774 cells or any other macrophage cell line and human intestinal epithelial Caco2 cells.

[0057] The nucleotide sequence of a nucleic acid may be determined by any method known in the art (*e.g.*, chemical sequencing or enzymatic sequencing). "Chemical sequencing" of DNA includes methods such as that of Maxam and Gilbert (1977) (Proc. Natl. Acad. Sci. USA 74: 560), in which DNA is randomly cleaved using individual base-specific reactions. "Enzymatic sequencing" of DNA includes methods such as that of Sanger (Sanger, *et al.*, (1977) Proc. Natl. Acad. Sci. USA 74: 5463).

[0058] The nucleic acids herein may be flanked by natural regulatory (expression control) sequences, or may be associated with heterologous sequences, including promoters, internal ribosome entry sites (IRES) and other ribosome binding site sequences, enhancers, response elements, suppressors, signal sequences, polyadenylation sequences, introns, 5'- and 3'- non-coding regions, and the like.

[0059] In general, a "promoter" or "promoter sequence" is a DNA regulatory region capable of binding an RNA polymerase in a cell (*e.g.*, directly or through other promoter-bound proteins or substances) and initiating transcription of a coding sequence. A promoter sequence is, in general, bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at any level. Within the promoter sequence may be found a transcription initiation site (conveniently defined, for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. The promoter may be operably associated with other expression control sequences, including enhancer and repressor sequences or with a nucleic acid of the invention. Promoters which may be used to control gene expression include, but are not limited to, cytomegalovirus (CMV) promoter (U.S. Patent Nos. 5,385,839 and 5,168,062), the SV40 early promoter region (Benoist, *et al.*, (1981) Nature 290: 304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, *et al.*, (1980) Cell 22: 787-797), the herpes thymidine kinase promoter (Wagner, *et al.*, (1981) Proc. Natl. Acad. Sci. USA 78: 1441-1445), the regulatory sequences of the metallothionein gene (Brinster, *et al.*, (1982) Nature 296: 39-42); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Komaroff, *et al.*, (1978) Proc. Natl. Acad. Sci. USA 75: 3727-3731), or the *tac* promoter (DeBoer, *et al.*, (1983) Proc. Natl. Acad. Sci. USA 80: 21-25); see also "Useful proteins from recombinant bacteria" in Scientific American (1980) 242: 74-94; and promoter elements from yeast or other fungi such as the *Gal 4* promoter, the *ADC* (alcohol dehydrogenase) promoter, *PGK* (phosphoglycerol kinase) promoter or the alkaline phosphatase promoter.

[0060] A coding sequence is "under the control of", "functionally associated with" or "operably associated with" transcriptional and translational control sequences in a cell when the sequences direct RNA polymerase mediated transcription of the coding sequence into RNA, preferably mRNA, which then may be RNA spliced (if it contains introns) and, optionally, translated into a protein encoded by the coding sequence.

[0061] The terms "express" and "expression" mean allowing or causing the information in a gene, RNA or DNA sequence to become manifest; for example, producing a protein by activating the cellular functions involved in transcription and translation of a corresponding gene. A DNA sequence is expressed in or by a cell to form an "expression product" such as an RNA (*e.g.*, mRNA) or a protein. The expression product itself may also be said to be "expressed" by the cell.

[0062] The term "transformation" means the introduction of a nucleic acid into a cell. The introduced gene or sequence may be called a "clone". A host cell that receives the introduced DNA or RNA has been "transformed" and is a "transformant" or a "clone." The DNA or RNA introduced to a host cell can come from any source, including cells of the same genus or species as the host cell, or from cells of a different genus or species.

[0063] The term "vector" includes a vehicle (*e.g.*, a plasmid) by which a DNA or RNA sequence can be introduced into a host cell, so as to transform the host and, optionally, promote expression and/or replication of the introduced sequence.

[0064] Vectors that can be used in this invention include plasmids, viruses, bacteriophage, integratable DNA fragments, and other vehicles that may facilitate introduction of the nucleic

acids into the genome of the host. Plasmids are the most commonly used form of vector but all other forms of vectors which serve a similar function and which are, or become, known in the art are suitable for use herein. See, *e.g.*, Pouwels, *et al.*, Cloning Vectors: A Laboratory Manual, 1985 and Supplements, Elsevier, N.Y., and Rodriguez *et al.* (eds.), Vectors: A Survey of Molecular Cloning Vectors and Their Uses, 1988, Buttersworth, Boston, MA.

[0065] The term "expression system" means a host cell and compatible vector which, under suitable conditions, can express a protein or nucleic acid which is carried by the vector and introduced to the host cell. Common expression systems include *E. coli* host cells and plasmid vectors, insect host cells and Baculovirus vectors, and mammalian host cells and vectors.

[0066] Expression of nucleic acids encoding the NPC1L1 polypeptides of this invention can be carried out by conventional methods in either prokaryotic or eukaryotic cells. Although *E. coli* host cells are employed most frequently in prokaryotic systems, many other bacteria, such as various strains of *Pseudomonas* and *Bacillus*, are known in the art and can be used as well. Suitable host cells for expressing nucleic acids encoding the NPC1L1 polypeptides include prokaryotes and higher eukaryotes. Prokaryotes include both gram-negative and gram-positive organisms, *e.g.*, *E. coli* and *B. subtilis*. Higher eukaryotes include established tissue culture cell lines from animal cells, both of non-mammalian origin, *e.g.*, insect cells, and birds, and of mammalian origin, *e.g.*, human, primates, and rodents.

[0067] Prokaryotic host-vector systems include a wide variety of vectors for many different species. A representative vector for amplifying DNA is pBR322 or many of its derivatives (*e.g.*, pUC18 or 19). Vectors that can be used to express the NPC1L1 polypeptides include, but are not limited to, those containing the *lac* promoter (pUC-series); *trp* promoter (pBR322-*trp*); *lpp*

promoter (the pIN-series); lambda-pP or pR promoters (pOTS); or hybrid promoters such as *ptac* (pDR540). See Brosius *et al.*, "Expression Vectors Employing Lambda-, *trp*-, lac-, and *Ipp*-derived Promoters", in Rodriguez and Denhardt (eds.) Vectors: A Survey of Molecular Cloning Vectors and Their Uses, 1988, Buttersworth, Boston, pp. 205-236. Many polypeptides can be expressed, at high levels, in an *E.coli*/T7 expression system as disclosed in U.S. Patent Nos. 4,952,496; 5,693,489 and 5,869,320 and in Davanloo, P., *et al.*, (1984) Proc. Natl. Acad. Sci. USA 81: 2035-2039; Studier, F.W., *et al.*, (1986) J. Mol. Biol. 189: 113-130; Rosenberg, A. H., *et al.*, (1987) Gene 56: 125-135; and Dunn, J.J., *et al.*, (1988) Gene 68: 259.

[0068] Higher eukaryotic tissue culture cells may also be used for the recombinant production of the NPC1L1 polypeptides of the invention. Although any higher eukaryotic tissue culture cell line might be used, including insect baculovirus expression systems, mammalian cells are preferred. Transformation or transfection and propagation of such cells have become a routine procedure. Examples of useful cell lines include HeLa cells, chinese hamster ovary (CHO) cell lines, J774 cells, Caco2 cells, baby rat kidney (BRK) cell lines, insect cell lines, bird cell lines, and monkey (COS) cell lines. Expression vectors for such cell lines usually include an origin of replication, a promoter, a translation initiation site, RNA splice sites (if genomic DNA is used), a polyadenylation site, and a transcription termination site. These vectors also, usually, contain a selection gene or amplification gene. Suitable expression vectors may be plasmids, viruses, or retroviruses carrying promoters derived, *e.g.*, from such sources as adenovirus, SV40, parvoviruses, vaccinia virus, or cytomegalovirus. Examples of expression vectors include pCR®3.1, pCDNA1, pCD (Okayama, *et al.*, (1985) Mol. Cell Biol. 5: 1136), pMC1neo Poly-A (Thomas, *et al.*, (1987) Cell 51: 503), pREP8, pSVSPORT and derivatives thereof, and

baculovirus vectors such as pAC373 or pAC610. One embodiment of the invention includes membrane bound NPC1L1. In this embodiment, NPC1L1 can be expressed in the cell membrane of a eukaryotic cell and the membrane bound protein can be isolated from the cell by conventional methods which are known in the art.

[0069] The present invention also includes fusions which include the NPC1L1 polypeptides and *NPC1L1* polynucleotides of the present invention and a second polypeptide or polynucleotide moiety, which may be referred to as a "tag". The fusions of the present invention may comprise any of the polynucleotides or polypeptides set forth in Table 1 or any subsequence or fragment thereof (discussed above). The fused polypeptides of the invention may be conveniently constructed, for example, by insertion of a polynucleotide of the invention or fragment thereof into an expression vector. The fusions of the invention may include tags which facilitate purification or detection. Such tags include glutathione-S-transferase (GST), hexahistidine (His6) tags, maltose binding protein (MBP) tags, haemagglutinin (HA) tags, cellulose binding protein (CBP) tags and myc tags. Detectable tags such as ^{32}P, ^{35}S, ^{3}H, ^{99m}Tc, ^{123}I, ^{111}In, ^{68}Ga, ^{18}F, ^{125}I, ^{131}I, ^{113m}In, ^{76}Br, ^{67}Ga, ^{99m}Tc, ^{123}I, ^{111}In and ^{68}Ga may also be used to label the polypeptides and polynucleotides of the invention. Methods for constructing and using such fusions are very conventional and well known in the art.

[0070] Modifications (*e.g.*, post-translational modifications) that occur in a polypeptide often will be a function of how it is made. For polypeptides made by expressing a cloned gene in a host, for instance, the nature and extent of the modifications, in large part, will be determined by the host cell's post-translational modification capacity and the modification signals present in the polypeptide amino acid sequence. For instance, as is well known, glycosylation often does not

occur in bacterial hosts such as *E. coli*. Accordingly, when glycosylation is desired, a polypeptide can be expressed in a glycosylating host, generally a eukaryotic cell. Insect cells often carry out post-translational glycosylations which are similar to those of mammalian cells. For this reason, insect cell expression systems have been developed to express, efficiently, mammalian proteins having native patterns of glycosylation. An insect cell which may be used in this invention is any cell derived from an organism of the class *Insecta*. Preferably, the insect is *Spodoptera fruigiperda* (Sf9 or Sf21) or *Trichoplusia ni* (High 5). Examples of insect expression systems that can be used with the present invention, for example to produce NPC1L1 polypeptide, include Bac-To-Bac (Invitrogen Corporation, Carlsbad, CA) or Gateway (Invitrogen Corporation, Carlsbad, CA). If desired, deglycosylation enzymes can be used to remove carbohydrates attached during production in eukaryotic expression systems.

[0071] Other modifications may also include addition of aliphatic esters or amides to the polypeptide carboxyl terminus. The present invention also includes analogs of the NPC1L1 polypeptides which contain modifications, such as incorporation of unnatural amino acid residues, or phosphorylated amino acid residues such as phosphotyrosine, phosphoserine or phosphothreonine residues. Other potential modifications include sulfonation, biotinylation, or the addition of other moieties. For example, the NPC1L1 polypeptides of the invention may be appended with a polymer which increases the half-life of the peptide in the body of a subject. Preferred polymers include polyethylene glycol (PEG) (*e.g.*, PEG with a molecular weight of 2 kDa, 5 kDa, 10 kDa, 12 kDa, 20 kDa, 30 kDa and 40 kDa), dextran and monomethoxypolyethylene glycol (mPEG).

[0072] The peptides of the invention may also be cyclized. Specifically, the amino- and carboxy-terminal residues of an NPC1L1 polypeptide or two internal residues of an NPC1L1 polypeptide of the invention can be fused to create a cyclized peptide. Methods for cyclizing peptides are conventional and very well known in the art; for example, see Gurrath, *et al.*, (1992) Eur. J. Biochem. 210: 911-921.

[0073] The present invention contemplates any superficial or slight modification to the amino acid or nucleotide sequences which correspond to the polypeptides of the invention. In particular, the present invention contemplates sequence conservative variants of the nucleic acids which encode the polypeptides of the invention. "Sequence-conservative variants" of a polynucleotide sequence are those in which a change of one or more nucleotides in a given codon results in no alteration in the amino acid encoded at that position. Function-conservative variants of the polypeptides of the invention are also contemplated by the present invention. "Function-conservative variants" are those in which one or more amino acid residues in a protein or enzyme have been changed without altering the overall conformation and function of the polypeptide, including, but, by no means, limited to, replacement of an amino acid with one having similar properties. Amino acids with similar properties are well known in the art. For example, polar/hydrophilic amino acids which may be interchangeable include asparagine, glutamine, serine, cysteine, threonine, lysine, arginine, histidine, aspartic acid and glutamic acid; nonpolar/hydrophobic amino acids which may be interchangeable include glycine, alanine, valine, leucine, isoleucine, proline, tyrosine, phenylalanine, tryptophan and methionine; acidic amino acids, which may be interchangeable include aspartic acid and glutamic acid and basic amino acids, which may be interchangeable include histidine, lysine and arginine.

[0074] The present invention includes polynucleotides encoding rat, human or mouse NPC1L1 and fragments thereof as well as nucleic acids which hybridize to the polynucleotides. Preferably, the nucleic acids hybridize under low stringency conditions, more preferably under moderate stringency conditions and most preferably under high stringency conditions. A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength (see Sambrook, *et al.*, supra). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. Typical low stringency hybridization conditions are 55°C, 5X SSC, 0.1% SDS, 0.25% milk, and no formamide at 42°C; or 30% formamide, 5X SSC, 0.5% SDS at 42°C. Typical, moderate stringency hybridization conditions are similar to the low stringency conditions except the hybridization is carried out in 40% formamide, with 5X or 6X SSC at 42°C. High stringency hybridization conditions are similar to low stringency conditions except the hybridization conditions are carried out in 50% formamide, 5X or 6X SSC and, optionally, at a higher temperature (*e.g.*, higher than 42°C: 57°C, 59°C, 60°C, 62°C, 63°C, 65°C or 68°C). In general, SSC is 0.15M NaCl and 0.015M Na-citrate. Hybridization requires that the two nucleic acids contain complementary sequences, although, depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the higher the stringency under which the nucleic acids may hybridize. For hybrids of greater than 100 nucleotides in length, equations for

calculating the melting temperature have been derived (see Sambrook, *et al.*, supra, 9.50-9.51). For hybridization with shorter nucleic acids, i.e., oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (see Sambrook, *et al.*, supra).

[0075] Also included in the present invention are polynucleotides comprising nucleotide sequences and polypeptides comprising amino acid sequences which are at least about 70% identical, preferably at least about 80% identical, more preferably at least about 90% identical and most preferably at least about 95% identical (*e.g.*, 95%, 96%, 97%, 98%, 99%, 100%) to the reference rat *NPC1L1* nucleotide (*e.g.*, any of SEQ ID NOs: 1 or 5-10) and amino acid sequences (*e.g.*, SEQ ID NO: 2), reference human *NPC1L1* nucleotide (*e.g.*, SEQ ID NO: 3) and amino acid sequences (*e.g.*, SEQ ID NO: 4) or the reference mouse *NPC1L1* nucleotide (*e.g.*, any of SEQ ID NOs: 11 or 13) and amino acid sequences (*e.g.*, SEQ ID NO: 12), when the comparison is performed by a BLAST algorithm wherein the parameters of the algorithm are selected to give the largest match between the respective sequences over the entire length of the respective reference sequences. Polypeptides comprising amino acid sequences which are at least about 70% similar, preferably at least about 80% similar, more preferably at least about 90% similar and most preferably at least about 95% similar (*e.g.*, 95%, 96%, 97%, 98%, 99%, 100%) to the reference rat NPC1L1 amino acid sequence of SEQ ID NO: 2, reference human NPC1L1 amino acid sequence of SEQ ID NO: 4 or the reference mouse NPC1L1 amino acid sequence of SEQ ID NO: 12, when the comparison is performed with a BLAST algorithm wherein the parameters of the algorithm are selected to give the largest match between the respective sequences over the entire length of the respective reference sequences, are also included in the present invention.

[0076] Sequence identity refers to exact matches between the nucleotides or amino acids of two sequences which are being compared. Sequence similarity refers to both exact matches between the amino acids of two polypeptides which are being compared in addition to matches between nonidentical, biochemically related amino acids. Biochemically related amino acids which share similar properties and may be interchangeable are discussed above.

[0077] The following references regarding the BLAST algorithm are herein incorporated by reference: **BLAST ALGORITHMS**: Altschul, S.F., *et al.*, (1990) J. Mol. Biol. 215: 403-410; Gish, W., *et al.*, (1993) Nature Genet. 3: 266-272; Madden, T.L., *et al.*, (1996) Meth. Enzymol. 266: 131-141; Altschul, S.F., *et al.*, (1997) Nucleic Acids Res. 25: 3389-3402; Zhang, J., *et al.*, (1997) Genome Res. 7: 649-656; Wootton, J.C., *et al.*, (1993) Comput. Chem. 17: 149-163; Hancock, J.M., *et al.*, (1994) Comput. Appl. Biosci. 10: 67-70; **ALIGNMENT SCORING SYSTEMS**: Dayhoff, M.O., *et al.*, "A model of evolutionary change in proteins" in Atlas of Protein Sequence and Structure, (1978) vol. 5, suppl. 3. M.O. Dayhoff (ed.), pp. 345-352, Natl. Biomed. Res. Found., Washington, DC; Schwartz, R.M., *et al.*, "Matrices for detecting distant relationships" in Atlas of Protein Sequence and Structure, (1978) vol. 5, suppl. 3. M.O. Dayhoff (ed.), pp. 353-358, Natl. Biomed. Res. Found., Washington, DC; Altschul, S.F., (1991) J. Mol. Biol. 219: 555-565; States, D.J., *et al.*, (1991) Methods 3: 66-70; Henikoff, S., *et al.*, (1992) Proc. Natl. Acad. Sci. USA 89: 10915-10919; Altschul, S.F., *et al.*, (1993) J. Mol. Evol. 36: 290-300; **ALIGNMENT STATISTICS**: Karlin, S., *et al.*, (1990) Proc. Natl. Acad. Sci. USA 87: 2264-2268; Karlin, S., *et al.*, (1993) Proc. Natl. Acad. Sci. USA 90: 5873-5877; Dembo, A., *et al.*, (1994) Ann. Prob. 22: 2022-2039; and Altschul, S.F. "Evaluating the statistical significance

of multiple distinct local alignments" in Theoretical and Computational Methods in Genome Research (S. Suhai, ed.), (1997) pp. 1-14, Plenum, New York.

Protein Purification

[0078] The proteins, polypeptides and antigenic fragments of this invention can be purified by standard methods, including, but not limited to, salt or alcohol precipitation, affinity chromatography (*e.g.*, used in conjunction with a purification tagged NPC1L1 polypeptide as discussed above), preparative disc-gel electrophoresis, isoelectric focusing, high pressure liquid chromatography (HPLC), reversed-phase HPLC, gel filtration, cation and anion exchange and partition chromatography, and countercurrent distribution. Such purification methods are well known in the art and are disclosed, *e.g.*, in "Guide to Protein Purification", Methods in Enzymology, Vol. 182, M. Deutscher, Ed., 1990, Academic Press, New York, NY.

[0079] Purification steps can be followed by performance of assays for receptor binding activity as described below. Particularly where an NPC1L1 polypeptide is being isolated from a cellular or tissue source, it is preferable to include one or more inhibitors of proteolytic enzymes in the assay system, such as phenylmethanesulfonyl fluoride (PMSF), Pefabloc SC, pepstatin, leupeptin, chymostatin and EDTA.

Antibody Molecules

[0080] Antigenic (including immunogenic) fragments of the NPC1L1 polypeptides of the invention are within the scope of the present invention (*e.g.*, 42 or more contiguous amino acids from SEQ ID NO: 2, 4 or 12). The antigenic peptides may be useful, *inter alia*, for preparing isolated antibody molecules which recognize NPC1L1. Isolated anti-NPC1L1 antibody molecules are useful NPC1L1 ligands.

[0081] An antigen is any molecule that can bind specifically to an antibody. Some antigens cannot, by themselves, elicit antibody production. Those that can induce antibody production are immunogens.

[0082] Preferably, isolated anti-NPC1L1 antibodies recognize an antigenic peptide comprising an amino acid sequence selected from SEQ ID NOs: 39-42 (*e.g.*, an antigen derived from rat NPC1L1). More preferably, the antibody is A0715, A0716, A0717, A0718, A0867, A0868, A1801 or A1802.

[0083] The term "antibody molecule " includes, but is not limited to, antibodies and fragments (preferably antigen-binding fragments) thereof. The term includes monoclonal antibodies, polyclonal antibodies, bispecific antibodies, Fab antibody fragments, $F(ab)_2$ antibody fragments, Fv antibody fragments (*e.g.*, V_H or V_L), single chain Fv antibody fragments and dsFv antibody fragments. Furthermore, the antibody molecules of the invention may be fully human antibodies, mouse antibodies, rat antibodies, rabbit antibodies, goat antibodies, chicken antibodies, humanized antibodies or chimeric antibodies.

[0084] Although it is not always necessary, when NPC1L1 polypeptides are used as antigens to elicit antibody production in an immunologically competent host, smaller antigenic fragments are, preferably, first rendered more immunogenic by cross-linking or concatenation, or by coupling to an immunogenic carrier molecule (i.e., a macromolecule having the property of independently eliciting an immunological response in a host animal, such as diptheria toxin or tetanus). Cross-linking or conjugation to a carrier molecule may be required because small polypeptide fragments sometimes act as haptens (molecules which are capable of specifically binding to an antibody but incapable of eliciting antibody production, i.e., they are not

immunogenic). Conjugation of such fragments to an immunogenic carrier molecule renders them more immunogenic through what is commonly known as the "carrier effect".

[0085] Carrier molecules include, *e.g.*, proteins and natural or synthetic polymeric compounds such as polypeptides, polysaccharides, lipopolysaccharides, etc. Protein carrier molecules are especially preferred, including, but not limited to, keyhole limpet hemocyanin and mammalian serum proteins such as human or bovine gammaglobulin, human, bovine or rabbit serum albumin, or methylated or other derivatives of such proteins. Other protein carriers will be apparent to those skilled in the art. Preferably, the protein carrier will be foreign to the host animal in which antibodies against the fragments are to be elicited.

[0086] Covalent coupling to the carrier molecule can be achieved using methods well known in the art, the exact choice of which will be dictated by the nature of the carrier molecule used. When the immunogenic carrier molecule is a protein, the fragments of the invention can be coupled, *e.g.*, using water-soluble carbodiimides such as dicyclohexylcarbodiimide or glutaraldehyde.

[0087] Coupling agents, such as these, can also be used to cross-link the fragments to themselves without the use of a separate carrier molecule. Such cross-linking into aggregates can also increase immunogenicity. Immunogenicity can also be increased by the use of known adjuvants, alone or in combination with coupling or aggregation.

[0088] Adjuvants for the vaccination of animals include, but are not limited to, Adjuvant 65 (containing peanut oil, mannide monooleate and aluminum monostearate); Freund's complete or incomplete adjuvant; mineral gels such as aluminum hydroxide, aluminum phosphate and alum;

surfactants such as hexadecylamine, octadecylamine, lysolecithin, dimethyldioctadecylammonium bromide, N,N-dioctadecyl-N',N'-bis(2-hydroxymethyl) propanediamine, methoxyhexadecylglycerol and pluronic polyols; polyanions such as pyran, dextran sulfate, poly IC, polyacrylic acid and carbopol; peptides such as muramyl dipeptide, dimethylglycine and tuftsin; and oil emulsions. The polypeptides could also be administered following incorporation into liposomes or other microcarriers.

[0089] Information concerning adjuvants and various aspects of immunoassays are disclosed, *e.g.*, in the series by P. Tijssen, Practice and Theory of Enzyme Immunoassays, 3rd Edition, 1987, Elsevier, New York. Other useful references covering methods for preparing polyclonal antisera include Microbiology, 1969, Hoeber Medical Division, Harper and Row; Landsteiner, Specificity of Serological Reactions, 1962, Dover Publications, New York, and Williams, *et al.*, Methods in Immunology and Immunochemistry, Vol. 1, 1967, Academic Press, New York.

[0090] The anti-NPC1L1 antibody molecules of the invention preferably recognize human, mouse or rat NPC1L1; however, the present invention includes antibody molecules which recognize NPC1L1 from any species, preferably mammals (*e.g.*, cat, sheep or horse). The present invention also includes complexes comprising an NPC1L1 polypeptide of the invention and an anti-NPC1L1 antibody molecule. Such complexes can be made by simply contacting the antibody molecule with its cognate polypeptide.

[0091] Various methods may be used to make the antibody molecules of the invention. Human antibodies can be made, for example, by methods which are similar to those disclosed in U.S. Patent Nos. 5,625,126; 5,877,397; 6,255,458; 6,023,010 and 5,874,299.

[0092] Hybridoma cells which produce the monoclonal anti-NPC1L1 antibodies may be produced by methods which are commonly known in the art. These methods include, but are not limited to, the hybridoma technique originally developed by Kohler, *et al.*, (1975) (Nature 256: 495-497), as well as the trioma technique (Hering, *et al.*, (1988) Biomed. Biochim. Acta. 47: 211-216 and Hagiwara, *et al.*, (1993) Hum. Antibod. Hybridomas 4: 15), the human B-cell hybridoma technique (Kozbor, *et al.*, (1983) Immunology Today 4: 72 and Cote, *et al.*, (1983) Proc. Natl. Acad. Sci. U.S.A 80: 2026-2030), and the EBV-hybridoma technique (Cole, *et al.*, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96, 1985). ELISA may be used to determine if hybridoma cells are expressing anti-NPC1L1 antibodies.

[0093] The anti-NPC1L1 antibody molecules of the present invention may also be produced recombinantly (*e.g.*, in an *E.coli*/T7 expression system as discussed above). In this embodiment, nucleic acids encoding the antibody molecules of the invention (*e.g.*, V_H or V_L) may be inserted into a pet-based plasmid and expressed in the *E.coli*/T7 system. There are several methods by which to produce recombinant antibodies which are known in the art. An example of a method for recombinant production of antibodies is disclosed in U.S. Patent No. 4,816,567. See also Skerra, A., *et al.*, (1988) Science 240: 1038-1041; Better, M., *et al.*, (1988) Science 240: 1041-1043 and Bird, R.E., *et al.*, (1988) Science 242: 423-426.

[0094] The term "monoclonal antibody," includes an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible, naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Monoclonal antibodies are advantageous in that they may be synthesized by a hybridoma

culture, essentially uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. The monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method as described by Kohler, *et al.*, (1975) Nature 256: 495.

[0095] The term "polyclonal antibody" includes an antibody which was produced among or in the presence of one or more other, non-identical antibodies. In general, polyclonal antibodies are produced from a B-lymphocyte in the presence of several other B-lymphocytes which produced non-identical antibodies. Typically, polyclonal antibodies are obtained directly from an immunized animal (*e.g.*, a rabbit).

[0096] A "bispecific antibody" comprises two different antigen binding regions which bind to distinct antigens. Bispecific antibodies, as well as methods of making and using the antibodies, are conventional and very well known in the art.

[0097] Anti-idiotypic antibodies or anti-idiotypes are antibodies directed against the antigen-combining region or variable region (called the idiotype) of another antibody molecule. As disclosed by Jerne (Jerne, N. K., (1974) Ann. Immunol. (Paris) 125c: 373 and Jerne, N. K., *et al.*, (1982) EMBO 1: 234), immunization with an antibody molecule expressing a paratope (antigen-combining site) for a given antigen (*e.g.*, NPC1L1) will produce a group of anti-antibodies, some of which share, with the antigen, a complementary structure to the paratope. Immunization with a subpopulation of the anti-idiotypic antibodies will, in turn, produce a subpopulation of antibodies or immune cell subsets that are reactive to the initial antigen.

[0098] The term "fully human antibody" refers to an antibody which comprises human immunoglobulin sequences only. Similarly, "mouse antibody" refers to an antibody which comprises mouse immunoglobulin sequences only.

[0100] "Human/mouse chimeric antibody" refers to an antibody which comprises a mouse variable region (V_H and V_L) fused to a human constant region.

[0101] "Humanized" anti-NPC1L1 antibodies are also within the scope of the present invention. Humanized forms of non-human (*e.g.*, murine) antibodies are chimeric immunoglobulins, which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region of the recipient are replaced by residues from a complementary determining region of a nonhuman species (donor antibody), such as mouse, rat or rabbit, having a desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are also replaced by corresponding non-human residues.

[0102] "Single-chain Fv" or "sFv" antibody fragments include the V_H and/or V_L domains of an antibody, wherein these domains are present in a single polypeptide chain. Generally, the sFv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. Techniques described for the production of single chain antibodies (U.S. Patent Nos. 5,476,786; 5,132,405 and 4,946,778) can be adapted to produce anti-NPC1L1 specific, single chain antibodies. For a review of sFv see Pluckthun in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore, eds., Springer-Verlag, N.Y., pp. 269-315 (1994).

[0103] "Disulfide stabilized Fv fragments" and "dsFv" include molecules having a variable heavy chain (V_H) and/or a variable light chain (V_L) which are linked by a disulfide bridge.

[0104] Antibody fragments within the scope of the present invention also include $F(ab)_2$ fragments which may be produced by enzymatic cleavage of an IgG by, for example, pepsin. Fab fragments may be produced by, for example, reduction of $F(ab)_2$ with dithiothreitol or mercaptoethylamine.

[0105] An FV fragment is a V_L or V_H region.

[0106] Depending on the amino acid sequences of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are at least five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), *e.g.*, IgG-1, IgG-2, IgG-3 and IgG-4; IgA-1 and IgA-2.

[0107] The anti-NPC1L1 antibody molecules of the invention may also be conjugated to a chemical moiety. The chemical moiety may be, *inter alia,* a polymer, a radionuclide or a cytotoxic factor. Preferably, the chemical moiety is a polymer which increases the half-life of the antibody molecule in the body of a subject. Suitable polymers include, but are by no means limited to, polyethylene glycol (PEG) (*e.g.*, PEG with a molecular weight of 2kDa, 5kDa, 10kDa, 12kDa, 20kDa, 30kDa or 40kDa), dextran and monomethoxypolyethylene glycol (mPEG). Methods for producing PEGylated anti-IL8 antibodies which are described in U.S. Patent No. 6,133,426 can be applied to the production of PEGylated anti-NPC1L1 antibodies of the invention. Lee, *et al.*, (1999) (Bioconj. Chem. 10: 973-981) discloses PEG conjugated single-chain antibodies. Wen, *et al.*, (2001) (Bioconj. Chem. 12: 545-553) discloses conjugating

antibodies with PEG which is attached to a radiometal chelator (diethylenetriaminpentaacetic acid (DTPA)).

[0108] The antibody molecules of the invention may also be conjugated with labels such as ^{99}Tc, ^{90}Y, ^{111}In, ^{32}P, ^{14}C, ^{125}I, ^{3}H, ^{131}I, ^{11}C, ^{15}O, ^{13}N, ^{18}F, ^{35}S, ^{51}Cr, ^{57}To, ^{226}Ra, ^{60}Co, ^{59}Fe, ^{57}Se, ^{152}Eu, ^{67}CU, ^{217}Ci, ^{211}At, ^{212}Pb, ^{47}Sc, ^{109}Pd, ^{234}Th, ^{40}K, ^{157}Gd, ^{55}Mn, ^{52}Tr or ^{56}Fe.

[0109] The antibody molecules of the invention may also be conjugated with fluorescent or chemilluminescent labels, including fluorophores such as rare earth chelates, fluorescein and its derivatives, rhodamine and its derivatives, isothiocyanate, phycoerythrin, phycocyanin, allophycocyanin, o-phthaladehyde, fluorescamine, ^{152}Eu, dansyl, umbelliferone, luciferin, luminal label, isoluminal label, an aromatic acridinium ester label, an imidazole label, an acridimium salt label, an oxalate ester label, an aequorin label, 2,3-dihydrophthalazinediones, biotin/avidin, spin labels and stable free radicals.

[0110] The antibody molecules may also be conjugated to a cytotoxic factor such as diptheria toxin, *Pseudomonas aeruginosa* exotoxin A chain, ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins and compounds (*e.g.*, fatty acids), dianthin proteins, *Phytoiacca americana* proteins PAPI, PAPII, and PAP-S, *momordica charantia* inhibitor, curcin, crotin, *saponaria officinalis* inhibitor, mitogellin, restrictocin, phenomycin, and enomycin.

[0111] Any method known in the art for conjugating the antibody molecules of the invention to the various moieties may be employed, including those methods described by Hunter, *et al.*, (1962) Nature 144: 945; David, *et al.*, (1974) Biochemistry 13: 1014; Pain, *et al.*, (1981) J. Immunol. Meth. 40: 219; and Nygren, J., (1982) Histochem. and Cytochem. 30: 407.

[0112] Methods for conjugating antibodies are conventional and very well known in the art.

Screening Assays

[0113] The invention allows the identification of selective ligands of NPC1L1 (*e.g.*, SEQ ID NO: 2, 4 or 12) that may be useful in treatment and management of a variety of medical conditions, including elevated serum sterol (*e.g.*, cholesterol) or 5α-stanol. Thus, NPC1L1 of this invention can be employed in screening systems to identify ligands. These ligands may be agonists or antagonists of NPC1L1. Essentially, these assays provide methods for identifying ligands of NPC1L1 by using (1) NPC1L1, (2) an appropriate known NPC1L1 ligand, agonist or antagonist, for example, a sterol (such as cholesterol, phytosterols, including, but not limited to, sitosterol, campesterol, stigmasterol and avenosterol), a cholesterol oxidation product, a 5α-stanol (including, but not limited to, cholestanol, 5α-campestanol and 5α-sitostanol), a substituted azetidinone (*e.g.*, ezetimibe), BODIPY-ezetimibe (Altmann, *et al.*, (2002) Biochim. Biophys. Acta 1580(1): 77-93) or 4", 6"-bis[(2-fluorophenyl)carbamoyl]-beta-D-cellobiosyl derivative of 11-ketotigogenin as described in DeNinno, *et al.*, (1997) (J. Med. Chem. 40(16): 2547-54) or any substituted azetidinone, and (3) a sample to be tested for the presence of a candidate NPC1L1 ligand.

[0114] The term "specific" when used to describe binding of, for example, a ligand of NPC1L1 in a screening assay is a term of art which refers to the extent by which the ligand or antagonist (*e.g.*, substituted azetidinone, ezetimibe, sterol (such as cholesterol) or 5α-stanol) binds preferentially to NPC1L1 in comparison to other proteins in the assay system. For example, detection of the specific binding of a ligand of NPC1L1 binds specifically to NPC1L1

is made apparent when a signal generated in the assay to indicate such binding exceeds, to any extent, a signal generated in a negative control wherein, for example, NPC1L1 or ligand is absent. Furthermore, "specific binding" includes binding of a ligand either directly to NPC1L1 or indirectly, for example via another moiety, in a complex of which NPC1L1 is a part. The moiety to which an NPC1L1 ligand binds can be another protein or a post-translational modification of NPC1L1 (*e.g.*, a lipid chain or a carbohydrate chain).

[0115] Non-limiting examples of suitable substituted azetidinones for use in the screening assays include those disclosed in U.S. Patent Nos. RE37,721; 5,631,365; 5,767,115; 5,846,966; 5,688,990; 5,656,624; 5,624,920; 5,698,548; 5,756,470; 5,688,787; 5,306,817; 5,633,246; 5,627,176; 5,688,785; 5,744,467; 5,846,966; 5,728,827; 6,632,933 and U.S. Patent Application Publication No 2003/0105028-each of which is herein incorporated by reference in its entirety.

[0116] The present invention provides for a method by which to evaluate whether a sample contains an NPC1L1 ligand by determining whether the sample contains a candidate compound which competes for binding between the known ligand (*e.g.*, ezetimibe) and NPC1L1. The ligand may be an agonist or antagonist.

[0117] Ezetimibe can be prepared by a variety of methods well know to those skilled in the art, for example such as are disclosed in U.S. Patents Nos. 5,631,365, 5,767,115, 5,846,966, 6,207,822, U.S. Patent Application Publication No. 2002/0193607 and PCT Patent Application WO 93/02048, each of which is incorporated herein by reference in its entirety.

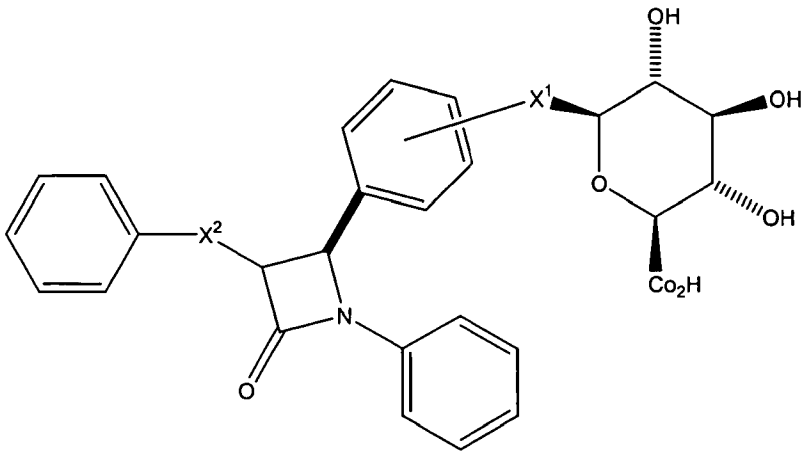
[0118] "Sample", "candidate compound" or "candidate substance" refers to a compound or composition which is evaluated in a test or assay, for example, for the ability to bind to NPC1L1

(*e.g.*, SEQ ID NO: 2, 4 or 12) or a functional fragment thereof. The composition may comprise candidate compounds, such as small molecules, peptides, nucleotides, polynucleotides, subatomic particles (*e.g.*, α particles, β particles) or antibodies.

[0119] The present invention provides methods for identifying ligands of a compound that binds to NPC1L1 which involve contacting NPC1L1 with a detectably labeled substituted 2-azetidinone, preferably substituted 2-azetidinone-glucuronide, and a candidate compound, and determining whether the candidate compound binds to NPC1L1. The modulation of the binding of the substituted 2-azetidinone to NPC1L1 by the binding of the candidate compound to NPC1L1 indicates that the candidate compound is a ligand that binds to NPC1L1 and is an inhibitor of sterol and 5α-stanol absorption.

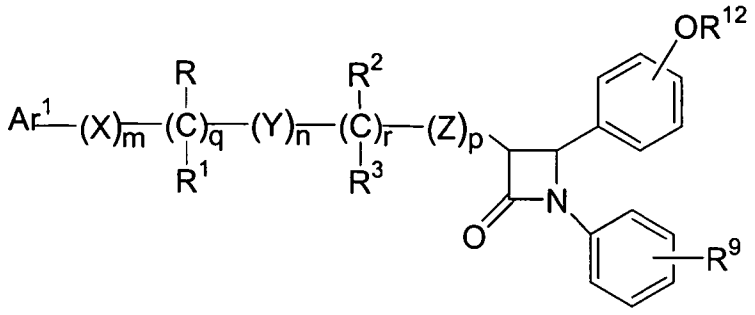
[0120] The present invention also provides a method for identifying a ligand of NPC1L1 comprising contacting NPC1L1 with a detectably labeled substituted 2-azetidinone, preferably substituted 2-azetidinone-glucuronide, and measuring the binding of NPC1L1 of the detectably labeled substituted 2-azetidinone in the presence and absence of a candidate compound, wherein decreased binding of the detectably labeled substituted 2-azetidinone to the NPC1L1 in the presence of the candidate compound indicates that said candidate compound is a ligand of NPC1L1 and is an inhibitor of sterol and 5α-stanol absorption.

[0121] Preferably, the substituted 2-azetidinones are detectably labelled with ^{3}H, ^{35}S, ^{125}I, or a fluorescently labeled substituted 2-azetidinone, preferably substituted 2-azetidinone-glucuronide. Compounds that are substituted 2-azetidinone-glucuronides are those having the following structure (I):



(I)

wherein X represents a group that links the glucuronide to the 4-phenyl ring, for example but not limited to –O- or $-C_{1-3}$ alkyl-, X^2 represents an optionally substituted –alkanediyl-, and wherein any of the phenyl groups may be optionally substituted. Examples of the phenyl-X^2-moiety in structure (I) include those represented at the 4-position on the 2-azetidinone structure shown below in structure (II). Additional examples of substituted 2-azetidinone-glucuronides include but are not limited to those described in U.S. Patent No. 5,756,470, WO02/066464 and US 2002/0137689. Additional examples of substituted 2-azetidinone-glucuronide compounds include those having the structure (II) and pharmaceutically acceptable salts and esters thereof as follows:



(II)

wherein:

Ar^1 is selected from the group consisting of aryl and R^4 -substituted aryl;

X, Y and Z are independently selected from the group consisting of $-CH_2-$, $-CH(C_{1-6}alkyl)-$ and $-C(C_{1-6}alkyl)_2-$;

R is selected from the group consisting of $-OR^6$, $-O(CO)R^6$, $-O(CO)OR^9$, $-O(CO)NR^6R^7$, a sugar residue, a disugar residue, a trisugar residue and a tetrasugar residue;

R^1 is selected from the group consisting of -H, $-C_{1-6}alkyl$ and aryl, or R and R^1 together are oxo;

R^2 is selected from the group consisting of $-OR^6$, $-O(CO)R^6$, $-O(CO)OR^9$ and $-O(CO)NR^6R^7$;

R^3 is selected from the group consisting of -H, $-C_{1-6}alkyl$ and aryl or R^2 and R^3 together are oxo;

q, r and t are each independently selected from 0 and 1;

m, n and p are each independently selected from 0, 1, 2, 3 and 4;

R^4 is 1-5 substituents independently selected at each occurrence from the group consisting of:

$-OR^5$, $-O(CO)R^5$, $-O(CO)OR^8$, $-O-C_{1-5}alkyl-OR^5$, $-O(CO)NR^5R^6$, $-NR^5R^6$, $-NR^5(CO)R^6$, $-NR^5(CO)OR^8$, $-NR^5(CO)NR^6R^7$, $-NR^5SO_2R^8$, $-COOR^5$, $-CONR^5R^6$, $-COR^5$, $-SO_2NR^5R^6$, $-S(O)_tR^8$, $-O-C_{1-10}alkyl-COOR^5$, $-O-C_{1-10}alkyl-CONR^5R^6$ and fluoro;

R^5, R^6 and R^7 are independently selected at each occurrence from the group consisting of -H, C_{1-6}alkyl, aryl and aryl-substituted C_{1-6}alkyl;

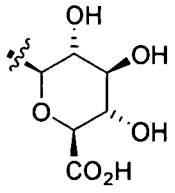
R^8 is independently selected from the group consisting of C_{1-6}alkyl, aryl and aryl-substituted C_{1-6}alkyl;

R^9 is selected from the group consisting of $-C\equiv C-CH_2-NR^{10}R^{11}$, $-C\equiv C-C(O)R^{13}$, and $-(CH_2)_3-NR^{10}R^{14}$;

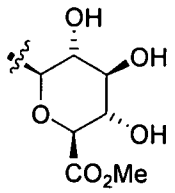
R^{10} is independently selected at each occurrence from –H and $-C_{1-3}$alkyl;

R^{11} is selected from the group consisting of –H, $-C_{1-3}$alkyl, $-C(O)-C_{1-3}$alkyl, $-C(O)-NR^{10}R^{10}$, $-SO_2-C_{1-3}$alkyl, and $-SO_2$-phenyl; and

R^{12} is selected from



and



(referred to herein as "glucuronide") (referred to herein as "methyl ester glucuronide");

R^{13} is selected from the group consisting of –OH and $-NR^{10}R^{11}$; and

R^{14} is selected from the group consisting of -C(O)-C_{1-3}alkyl, -C(O)-$NR^{10}R^{10}$, -SO_2-C_{1-3}alkyl and -SO_2-phenyl.

[0122] The above compound (II) is described in U.S. Provisional Application No._____, filed Dec 13, 2003.

[0123] Two additional types of screening systems that can be used include a labeled-ligand binding assay (*e.g.*, direct binding assay or scintillation proximity assay (SPA)) and a "sterol (*e.g.*, cholesterol) or 5α-stanol uptake" assay. A labeled ligand, for use in the binding assay, can be obtained by labeling a sterol (*e.g.*, cholesterol) or a 5α-stanol or a known NPC1L1 agonist or antagonist with a measurable group (*e.g.*, ^{35}S, ^{125}I or ^{3}H). Various labeled forms of sterols (*e.g.*, cholesterol) or 5α-stanols are available commercially or can be generated using standard techniques (*e.g.*, Cholesterol- [1,2-$^{3}H(N)$], Cholesterol-[1,2,6,7-$^{3}H(N)$] or Cholesterol-[7-$^{3}H(N)$]; American Radiolabeled Chemicals, Inc; St. Louis, MO). In a preferred embodiment, ezetimibe is fluorescently labeled with a BODIPY group (Altmann, *et al.*, (2002) Biochim. Biophys. Acta 1580(1): 77-93) or labeled with a detectable group such as ^{35}S, ^{125}I or ^{3}H.

[0124] ***Direct Binding Assay.*** Typically, a given amount of NPC1L1 of the invention (*e.g.*, SEQ ID NO: 2, 4 or 12) or a complex including NPC1L1 is contacted with increasing amounts of labeled ligand or known antagonist or agonist (discussed above) and the amount of the bound, labeled ligand or known antagonist or agonist is measured after removing unbound, labeled ligand or known antagonist or agonist by washing. As the amount of the labeled ligand or known agonist or antagonist is increased, a point is eventually reached at which all receptor binding sites are occupied or saturated. Specific receptor binding of the labeled ligand or known

agonist or antagonist is abolished by a large excess of unlabeled ligand or known agonist or antagonist.

[0125] Preferably, an assay system is used in which non-specific binding of the labeled ligand or known antagonist or agonist to the receptor is minimal. Non-specific binding is typically less than 50%, preferably less than 15%, and more preferably less than 10% of the total binding of the labeled ligand or known antagonist or agonist.

[0126] In the basic binding assay, the method for identifying an NPC1L1 ligand, agonist or antagonist includes:

(a) contacting NPC1L1 (*e.g.*, SEQ ID NO: 2 or 4 or 12), a fragment thereof or a complex including NPC1L1, in the presence of a known amount of labeled sterol (*e.g.*, cholesterol) or 5α-stanol or known antagonist or agonist (*e.g.*, labeled ezetimibe) with a sample to be tested for the presence of an NPC1L1 ligand, agonist or antagonist; and

(b) measuring the amount of labeled sterol (*e.g.*, cholesterol) or 5α-stanol or known antagonist or agonist directly or indirectly bound to NPC1L1.

[0127] An NPC1L1 ligand in the sample is identified by measuring substantially reduced direct or indirect binding of the labeled sterol (*e.g.*, cholesterol) or 5α-stanol or known antagonist or agonist to NPC1L1, compared to what would be measured in the absence of such a ligand. For example, reduced direct or indirect binding between [^{3}H]-cholesterol and NPC1L1 in the presence of a sample might suggest that the sample contains a substance which is competing against [^{3}H]-cholesterol for NPC1L1 binding.

[0128] This assay can include a control experiment lacking any NPC1L1-dependent ligand (*e.g.*, sterol such as cholesterol or 5α-stanol) binding. In this assay, for example, a whole cell or cell membrane lacking any functional NPC1L1, for example, a cell or membrane isolated or derived from a transgenic mutant *npc1l1*$^-$ mouse of the invention, is assayed for ligand binding. When screening a sample for the presence of an NPC1L1 antagonist, it is useful to compare the level of binding observed in the presence of a sample being tested with that of a control experiment, as described herein, which completely lacks NPC1L1-dependent binding. Ideally, though by no means necessarily, the level of binding seen in the presence of a sample containing an antagonist will be similar to that of the control experiment.

[0129] Alternatively, a sample can be tested directly for binding to NPC1L1 (*e.g.*, SEQ ID NO: 2, 4 or 12). A basic assay of this type may include the following steps:

(a) contacting NPC1L1 (*e.g.*, SEQ ID NO: 2 or 4 or 12), a fragment thereof or a complex including NPC1L1 with a labeled candidate compound (*e.g.*, [^{3}H]-ezetimibe); and

(b) detecting direct or indirect binding between the labeled candidate compound and NPC1L1.

[0130] Again, these experiment can be performed along with a control experiment wherein NPC1L1-dependent binding is completely lacking. For example, the assay can be performed using a whole cell or cell membrane lacking any functional NPC1L1 (*e.g.*, cell or cell membrane derived from a transgenic, mutant *npc1l1*$^-$ mouse as described herein).

[0131] A candidate compound which is found to bind to NPC1L1 may function as ligand, agonist or antagonist of NPC1L1 (*e.g.*, by inhibition of sterol (*e.g.*, cholesterol) or 5α-stanol uptake).

[0132] ***SPA Assay.*** NPC1L1 ligands may also be measured using scintillation proximity assays (SPA). SPA assays are conventional and very well known in the art; see, for example, U.S. Patent No. 4,568,649. In SPA, the target of interest is immobilized to a small microsphere approximately 5 microns in diameter. The microsphere, typically, includes a solid scintillant core which has been coated with a polyhydroxy film, which in turn contains coupling molecules, which allow generic links for assay design. When a radioisotopically labeled molecule binds to the microsphere, the radioisotope is brought into close proximity to the scintillant and effective energy transfer from electrons emitted by the isotope will take place resulting in the emission of light. While the radioisotope remains in free solution, it is too distant from the scintillant and the electron will dissipate the energy into the aqueous medium and therefore remain undetected. Scintillation may be detected with a scintillation counter. In general, ^{3}H and ^{125}I labels are well suited to SPA.

[0133] For the assay of receptor-mediated binding events, the lectin wheat germ agglutinin (WGA) may be used as the SPA bead coupling molecule (Amersham Biosciences; Piscataway, NJ). The WGA coupled bead captures glycosylated, cellular membranes and glycoproteins and has been used for a wide variety of receptor sources and cultured cell membranes. The receptor is immobilized onto the WGA-SPA bead and a signal is generated on binding of an isotopically labeled ligand. Other coupling molecules which may be useful for receptor binding SPA assays

include poly-L-lysine and WGA/polyethyleneimine (Amersham Biosciences; Piscataway, NJ). See, for example, Berry, J.A., *et al.*, (1991) Cardiovascular Pharmacol. 17 (Suppl.7): S143-S145; Hoffman, R., *et al.*, (1992) Anal. Biochem. 203: 70-75; Kienhus, *et al.*, (1992) J. Receptor Research 12: 389-399; Jing, S., *et al.*, (1992) Neuron 9: 1067-1079.

[0134] The scintillant contained in SPA beads may include, for example, yttrium silicate (YSi), yttrium oxide (YOx), diphenyloxazole or polyvinyltoluene (PVT) which acts as a solid solvent for diphenylanthracine (DPA).

[0135] SPA assays may be used to analyze whether a sample contains an NPC1L1 ligand. In these assays, a host cell which expresses NPC1L1 (*e.g.*, SEQ ID NO: 2 or 4 or 12) on the cell surface or a membrane fraction thereof is incubated with and captured by SPA beads (*e.g.*, WGA coated YOx beads or WGA coated YSi beads). The beads bearing the NPC1L1 are incubated with labeled, known ligand or agonist or antagonist (*e.g.*, ^{3}H-cholesterol, ^{3}H-ezetimibe or ^{125}I-ezetimibe). The assay mixture further includes either the sample to be tested or a blank (*e.g.*, water). After an optional incubation, scintillation is measured using a scintillation counter. An NPC1L1 ligand, agonist or antagonist may be identified in the sample by measuring substantially reduced fluorescence, compared to what would be measured in the absence of such ligand, agonist or antagonist (blank). Measuring substantially reduced fluorescence may suggest that the sample contains a substance which competes for direct or indirect NPC1L1 binding with the known ligand, agonist or antagonist.

[0136] Alternatively, a sample may be identified as an ligand of NPC1L1 by directly detecting binding in a SPA assay. In this assay, a labeled version of a candidate compound to be

tested may be put in contact with the host cell expressing NPC1L1 or a membrane fraction thereof which is bound to the SPA bead. Fluorescence may then be assayed to detect the presence of a complex between the labeled candidate compound and the host cell or membrane fraction expressing NPC1L1 or a complex including NPC1L1. A candidate compound which binds directly or indirectly to NPC1L1 may possess NPC1L1 agonistic or antagonistic activity.

[0137] SPA Assays can also be performed along with a control experiment lacking any NPC1L1-dependent binding. The control experiment can be performed, for example, with a cell or cell membrane lacking any functional NPC1L1 (*e.g.*, cell or cell membrane derived from a transgenic, mutant npc1l1- mouse as described herein). When the control experiment is performed, the level of binding observed in the presence of sample being tested for the presence of an antagonist can be compared with that observed in the control experiment.

[0138] ***Sterol/5α-stanol Uptake Assay.*** Assays may also be performed to determine if a sample can agonize or antagonize NPC1L1 mediated sterol (*e.g.*, cholesterol) or 5α-stanol uptake. In these assays, a host cell expressing NPC1L1 (*e.g.*, SEQ ID NO: 2 or 4 or 12) on the cell surface (discussed above) can be contacted with detectably labeled sterol (*e.g.*, ^{3}H-cholesterol or ^{125}I-cholesterol)) or 5α-stanol along with either a sample or a blank. After an optional incubation, the cells can be washed to remove unabsorbed sterol or 5α-stanol. Sterol or 5α-stanol uptake can be determined by detecting the presence of labeled sterol or 5α-stanol in the host cells. For example, assayed cells or lysates or fractions thereof (*e.g.*, fractions resolved by thin-layer chromatography) can be contacted with a liquid scintillant and scintillation can be measured using a scintillation counter.

[0139] In these assays, an NPC1L1 antagonist in the sample may be identified by measuring substantially reduced uptake of labeled sterol (*e.g.*, ^{3}H-cholesterol) or 5α-stanol, compared to what would be measured in the absence of such an antagonist and an agonist may be identified by measuring substantially increased uptake of labeled sterol (*e.g.*, ^{3}H-cholesterol) or 5α-stanol, compared to what would be measured in the absence of such an agonist.

[0140] Uptake assays can also be performed along with a control experiment lacking any NPC1L1-dependent uptake. The control experiment can be performed, for example, with a cell lacking any functional NPC1L1 (*e.g.*, cell derived from a transgenic, mutant *npc1l1*$^{-}$ mouse as described herein). When the control experiment is performed, the level of uptake observed in the presence of sample being tested for the presence of an antagonist can be compared with that observed in the control experiment.

[0141] ***Source of NPC1L1.*** In principle, a binding assay of the invention could be carried out using a soluble NPC1L1 polypeptide of the invention, *e.g.*, following production and refolding by standard methods from an *E. coli* or other prokaryotic or eukaryotic expression system, and the resulting receptor-labeled ligand complex could be precipitated, *e.g.*, using an antibody against the receptor. The precipitate could then be washed and the amount of the bound, labeled ligand or antagonist or agonist could be measured.

[0142] Alternatively, a nucleic acid encoding an NPC1L1 polypeptide of the invention (*e.g.*, SEQ ID NO: 2, 4 or 12) can be transfected into an appropriate host cell, whereby the NPC1L1 will become incorporated into the membrane of the cell. A membrane fraction can then be isolated from the cell and used as a source of NPC1L1 for assay. Alternatively, the whole cell

expressing NPC1L1 in the cell surface can be used in an assay. Preferably, specific binding of the labeled ligand or known antagonist or agonist to an untransfected/untransformed host cell or to a membrane fraction from an untransfected/untransformed host cell will be negligible.

[0143] Various membranes may be used directly as a source of NPC1L1 for the above-described screening systems, e.g. direct binding, scintillation proximity assay, sterol/5α stanol uptake assay. As described in Examples 5, 6, 7, 8, 9, 17, 27, and 29, NPC1L1 is highly expressed in certain tissues, especially in brush border cells of intestinal tissues. Therefore, brush border membrane vesical preparations may be utilized as a source of NPC1L1. The membranes may be derived from mammalian intestinal tissue from rhesus, rat, mouse or human tissue.

[0144] Membranes may be derived from brush border cells of intestinal tissues. Such membranes are conventionally prepared by collecting intestinal tissue from freshly sacrificed animals. The mucosa of the tissue is scraped, collected into buffered solutions, and homogenized. Cellular debris is removed and the membrane fractions are collected by centrifugation. Conventional techniques known to one of skill in the art maybe used for the preparation of brush border membrane vesicules. *See* Hauser, H., Howell, K., Dawson, R.M.C., Bowyer, D. E. Biochim. Biophys. Acta 602, 567-577 (1980); Kramer, W., Girbig, F., Gutjahr, U., Kowalewski, S., Jouvenal, K., Muller, G., Tripier, D., Wess, G. J. Biol. Chem. 268, 18035-18046 (1993); Rigtrup, K.M., Ong, D.E. Biochemistry 31, 2920-2926 (1992).

[0145] The membrane preparation may be in vesicular or non-vesicular form.

[0146] Alternatively, liposomes and liposomal preparations comprising NPC1L1 may also be a viable source of NPC1L1 for the screening assays of the present claimed method.

[0147] *In vitro* cultured cells expressing NPC1L1 may also be used. The host cells may be prepared by transforming or transfecting a nucleic acid encoding an NPC1L1 of the invention into an appropriate host cell, whereby the receptor becomes incorporated into the membrane of the cell. A membrane fraction can then be isolated from the cell and used as a source of the receptor for assay. Alternatively, the whole cell expressing the receptor on the cell surface can be used in an assay. Preferably, specific binding of the labeled ligand or known antagonist or agonist to an untransfected/untransformed host cell or membrane fraction from an untransfected/untransformed host cell will be negligible.

[0148] Preferred host cells include Chinese Hamster Ovary (CHO) cells, murine macrophage J774 cells or any other macrophage cell line and human intestinal epithelial Caco2 cells.

[0149] The present invention provides for a method of identifying a ligand of NPC1L1 using these membrane preparations, for example by contacting membranes comprising NPC1L1, such as brush border membrane vesicle preparations, with detectably labeled substituted azetidinone compounds which are known NPC1L1 ligands, agonists or antagonists, and a candidate compound and determining whether the candidate compound can bind to NPC1L1. The binding of the candidate compound to NPC1L1 may modulate binding of the detectably labeled NPC1L1 ligands, agonists or antagonists to NPC1L1. In addition, a NPC1L1 ligand may be identified by measuring the binding of NPC1L1 with detectably labeled NPC1L1 ligands, agonists or antagonists in the presence and absence of the candidate compound wherein decreased binding

of the detectably labeled NPC1L1 ligands, agonists or antagonists to NPC1L1 is an indication that the candidate compound is ligand of NPC1L1.

[0150] NPC1L1 may also be obtained by solubilization of membrane fractions comprising NPC1L1. The membranes may be obtained as discussed above, *e.g.,* from mammalian tissue or *in vitro* cultured cells.

[0151] ***Mouse Assay.*** The present invention comprises a mutant, transgenic mouse which lacks any functional NPC1L1. This mouse may serve as a convenient control experiment in screening assays for identifying inhibitors of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption, preferably inhibitors of NPC1L1. Preferably, a mouse assay of the present invention would comprise the following steps:

(a) feeding a sterol (*e.g.*, cholesterol) or 5α-stanol-containing substance (*e.g.*, comprising radiolabeled cholesterol, such as ^{14}C-cholesterol or ^{3}H-cholesterol) to a first and second mouse comprising a functional *NPC1L1* gene and to a third, mutant mouse lacking a functional NPC1L1;

[0152] The sterol (*e.g.*, cholesterol) or 5α-stanol containing substance preferably contains labeled cholesterol, such as a radiolabeled cholesterol, for example, ^{3}H or ^{14}C labeled cholesterol. The sterol (*e.g.*, cholesterol) or 5α-stanol containing substance may also include cold, unlabeled sterol (*e.g.*, cholesterol) or 5α-stanol such as in corn oil.

[0153] In these assays, the third *npc1l1*$^{-}$ mutant mouse serves as a (+)-control experiment which exhibits low levels of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption and the

second mouse serves as a (-)-control experiment which exhibits normal, uninhibited levels of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption. The second mouse is not administered the sample to be tested for an NPC1L1 antagonist. The first mouse is the experiment.

(b) administering the sample to the first mouse comprising a functional *NPC1L1* but not to the second mouse;

(c) measuring the amount of sterol (*e.g.*, cholesterol) or 5α-stanol absorption in the intestine of said first, second and third mouse;

[0154] Intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption may be measured by any method known in the art. For example, the level intestinal absorption can be assayed by measuring the level of serum sterol (*e.g.*, cholesterol) or 5α-stanol.

(d) comparing the levels of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption in each mouse;

wherein the sample is determined to contain the intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption antagonist when the level of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption in the first mouse and in the third mouse are less than the amount of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption in the second mouse.

[0155] Preferably, if the sample contains an intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption inhibitor (*e.g.*, an NPC1L1 inhibitor), the level of sterol (*e.g.*, cholesterol) or 5α-stanol absorption in the first mouse will be similar to that of the third, *npc1l1* mutant mouse.

[0156] An alternative, (+)-control experiment which may be used in these screening assays is a mouse comprising functional NPC1L1 which is administered a known antagonist of NPC1L1, such as ezetimibe.

Pharmaceutical Compositions

[0157] NPC1L1 ligands discovered, for example, by the screening methods described above may be used therapeutically (*e.g.*, in a pharmaceutical composition) to stimulate or block the activity of NPC1L1 and, thereby, to treat any medical condition caused or mediated by NPC1L1. In addition, the antibody molecules of the invention may also be used therapeutically (*e.g.*, in a pharmaceutical composition) to bind NPC1L1 and, thereby, block the ability of NPC1L1 to bind a sterol (*e.g.*, cholesterol) or 5α-stanol. Blocking the binding of a sterol (*e.g.*, cholesterol) or 5α-stanol would prevent absorption of the molecule (*e.g.*, by intestinal cells such as enterocytes). Blocking absorption of sterol (*e.g.*, cholesterol) or 5α-stanol would be a useful way to lower serum sterol (*e.g.*, cholesterol) or 5α-stanol levels in a subject and, thereby, reduce the incidence of, for example, hyperlipidemia, atherosclerosis, coronary heart disease, stroke or arteriosclerosis.

[0158] The term "subject" or "patient" includes any organism, preferably animals, more preferably mammals (*e.g.*, mice, rats, rabbits, dogs, horses, primates, cats) and most preferably humans.

[0159] The term "pharmaceutical composition" refers to a composition including an active ingredient and a pharmaceutically acceptable carrier and/or adjuvant.

[0160] Although the compositions of this invention could be administered in simple solution, they are more typically used in combination with other materials such as carriers, preferably pharmaceutically acceptable carriers. Useful, pharmaceutically acceptable carriers can be any compatible, non-toxic substances suitable for delivering the compositions of the invention to a subject. Sterile water, alcohol, fats, waxes, and inert solids may be included in a pharmaceutically acceptable carrier. Pharmaceutically acceptable adjuvants (buffering agents, dispersing agents) may also be incorporated into the pharmaceutical composition.

[0161] Preferably, the pharmaceutical compositions of the invention are in the form of a pill or capsule. Methods for formulating pills and capsules are very well known in the art. For example, for oral administration in the form of tablets or capsules, the active drug component may be combined with any oral, non-toxic pharmaceutically acceptable inert carrier, such as lactose, starch, sucrose, cellulose, magnesium stearate, dicalcium phosphate, calcium sulfate, talc, mannitol, ethyl alcohol (liquid forms) and the like. Moreover, when desired or needed, suitable binders, lubricants, disintegrating agents and coloring agents may also be incorporated in the mixture. Suitable binders include starch, gelatin, natural sugars, corn sweeteners, natural and synthetic gums such as acacia, sodium alginate, carboxymethylcellulose, polyethylene glycol and waxes. Among the lubricants there may be mentioned for use in these dosage forms, boric acid, sodium benzoate, sodium acetate, sodium chloride, and the like. Disintegrants include starch, methylcellulose, guar gum and the like. Sweetening and flavoring agents and preservatives may also be included where appropriate.

[0162] The pharmaceutical compositions of the invention may be administered in conjunction with a second pharmaceutical composition or substance. In preferred embodiments, the second composition includes a cholesterol-lowering drug. When a combination therapy is used, both compositions may be formulated into a single composition for simultaneous delivery or formulated separately into two or more compositions (*e.g.*, a kit).

[0163] The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. See, *e.g.*, Gilman *et al.* (eds.) (1990), *The Pharmacological Bases of Therapeutics,* 8th Ed., Pergamon Press; and *Remington's Pharmaceutical Sciences, supra,* Easton, Penn.; Avis *et al.* (eds.) (1993) *Pharmaceutical Dosage Forms: Parenteral Medications* Dekker, New York; Lieberman *et al.* (eds.) (1990) *Pharmaceutical Dosage Forms: Tablets* Dekker, New York; and Lieberman *et al.* (eds.) (1990), *Pharmaceutical Dosage Forms: Disperse Systems* Dekker, New York.

[0164] The dosage regimen involved in a therapeutic application may be determined by a physician, considering various factors which may modify the action of the therapeutic substance, *e.g.*, the condition, body weight, sex and diet of the patient, the severity of any infection, time of administration, and other clinical factors. Often, treatment dosages are titrated upward from a low level to optimize safety and efficacy. Dosages may be adjusted to account for the smaller molecular sizes and possibly decreased half-lives (clearance times) following administration.

[0165] An "effective amount" of a ligand of the invention may be an amount that will detectably reduce the level of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption or

detectably reduce the level of serum sterol (*e.g.*, cholesterol) or 5α-stanol in a subject administered the composition.

[0166] Typical protocols for the therapeutic administration of such substances are well known in the art. Pharmaceutical composition of the invention may be administered, for example, by any parenteral or non-parenteral route.

[0167] Pills and capsules of the invention can be administered orally. Injectable compositions can be administered with medical devices known in the art; for example, by injection with a hypodermic needle.

[0168] Injectable pharmaceutical compositions of the invention may also be administered with a needleless hypodermic injection device; such as the devices disclosed in U.S. Patent Nos. 5,399,163; 5,383,851; 5,312,335; 5,064,413; 4,941,880; 4,790,824 or 4,596,556.

Anti-Sense

[0169] The present invention also encompasses anti-sense oligonucleotides capable of specifically hybridizing to mRNA encoding NPC1L1 (*e.g.*, any of SEQ ID NOs: 1, 3, 5-11 or 13) having an amino acid sequence defined by, for example, SEQ ID NO: 2 or 4 or 12 or a subsequence thereof so as to prevent translation of the mRNA. Additionally, this invention contemplates anti-sense oligonucleotides capable of specifically hybridizing to the genomic DNA molecule encoding NPC1L1, for example, having an amino acid sequence defined by SEQ ID NO: 2 or 4 or 12 or a subsequence thereof.

[0170] This invention further provides pharmaceutical compositions comprising (a) an amount of an oligonucleotide effective to reduce NPC1L1-mediated sterol (*e.g.*, cholesterol) or 5α-stanol absorption by passing through a cell membrane and binding specifically with mRNA encoding NPC1L1 in the cell so as to prevent its translation and (b) a pharmaceutically acceptable carrier capable of passing through a cell membrane. In an embodiment, the oligonucleotide is coupled to a substance that inactivates mRNA. In another embodiment, the substance that inactivates mRNA is a ribozyme.

[0171] Reducing the level of NPC1L1 expression by introducing anti-sense NPC1L1 RNA into the cells of a patient is a useful method reducing intestinal sterol (*e.g.*, cholesterol) or 5 - stanol absorption and serum cholesterol in the patient.

Kits

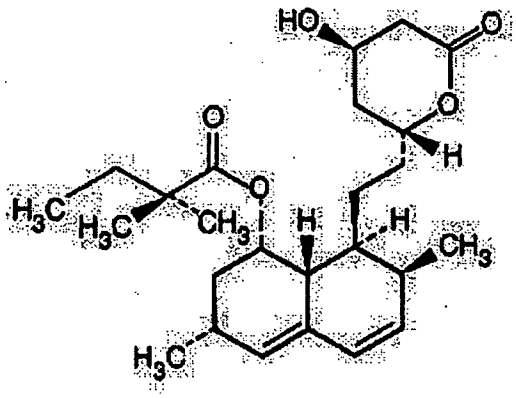
[0172] Kits of the present invention include ezetimibe, preferably combined with a pharmaceutically acceptable carrier, in a pharmaceutical formulation, more preferably in a pharmaceutical dosage form such as a pill, a powder, an injectable liquid, a tablet, dispersible granules, a capsule, a cachet or a suppository. See for example, Gilman *et al.* (eds.) (1990), The Pharmacological Bases of Therapeutics, 8th Ed., Pergamon Press; and Remington's Pharmaceutical Sciences, supra, Easton, Penn.; Avis *et al.* (eds.) (1993) Pharmaceutical Dosage Forms: Parenteral Medications Dekker, New York; Lieberman *et al.* (eds.) (1990) Pharmaceutical Dosage Forms: Tablets Dekker, New York; and Lieberman *et al.* (eds.) (1990), Pharmaceutical Dosage Forms: Disperse Systems Dekker, New York. Preferably, the dosage form is a Zetia® tablet (Merck/Schering-Plough Corp.). Ezetimibe may be supplied in any

convenient form. For example, tablets including ezetimibe may be supplied in bottles of 30, 90 or 500.

[0173] The kits of the present invention also include information, for example in the form of a package insert, indicating that the target of ezetimibe is NPC1L1 (NPC3). The term "target of ezetimibe" indicates that ezetimibe reduces intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption, either directly or indirectly, by antagonizing NPC1L1. The form of the insert may take any form, such as paper or on electronic media such as a magnetically recorded medium (*e.g.*, floppy disk) or a CD-ROM.

[0174] The package insert may also include other information concerning the pharmaceutical compositions and dosage forms in the kit. Generally, such information aids patients and physicians in using the enclosed pharmaceutical compositions and dosage forms effectively and safely. For example, the following information regarding ezetimibe (*e.g.*, Zetia®) and/or simvastatin (*e.g.*, Zocor®) may be supplied in the insert: pharmacokinetics, pharmacodynamics, clinical studies, efficacy parameters, indications and usage, contraindications, warnings, precautions, adverse reactions, overdosage, proper dosage and administration, how supplied, proper storage conditions, references and patent information.

[0175] The kits of the invention may also include simvastatin (



) preferably combined with a pharmaceutically acceptable carrier, in a pharmaceutical formulation, more preferably in a pharmaceutical dosage form such as a pill, a powder, an injectable liquid, a tablet, dispersible granules, a capsule, a cachet or a suppository. Preferably, the dosage form of simvastatin is a Zocor® tablet (Merck & Co.; Whitehouse Station, NJ).

[0099] Tablets or pills comprising simvastatin may be supplied in any convenient form. For example, pills or tablets comprising 5mg simvastatin can be supplied as follows: bottles of 30, 60, 90, 100 or 1000. Pills or tablets comprising 10 mg simvastatin may be supplied as follows: bottles of 30, 60, 90, 100, 1000 or 10,000. Pills or tablets comprising 20 mg simvastatin may be supplied as follows: bottles of 30, 60, 90, 100, 1000 or 10,000. Pills or tablets comprising 40 mg simvastatin may be supplied as follows: bottles of 30, 60, 90, 100 or 1000. Pills or tablets comprising 80 mg simvastatin may be supplied as follows: bottles of 30, 60, 90, 100, 1000 or 10,000.

[0176] Ezetimibe and simvastatin may be supplied, in the kit, as separate compositions or combined into a single composition. For example, ezetimibe and simvastatin may be supplied

within a single, common pharmaceutical dosage form (*e.g.*, pill or tablet) as in separate pharmaceutical dosage forms (*e.g.*, two separate pills or tablets).

npc1l1$^-$ Cells

[0177] The present invention provides any isolated mammalian cell, (*e.g.*, an isolated mouse cell, an isolated rat cell or an isolated human cell) which lacks an NPC1L1 gene which encodes or can produce a functional NPC1L1 protein. Included within this embodiment are mutant *npc1l1* genes comprising a point mutation, truncation or deletion of the genetic coding region or of any regulatory element (*e.g.*, a promoter).

[0178] For example, the cell can be isolated from a mutant mouse comprising a homozygous mutation of endogenous, chromosomal NPC1L1 wherein the mouse does not produce any functional NPC1L1 protein (*e.g.*, the mouse described below in Example 22). Moreover, the present invention comprises any cell, tissue, organ, fluid, nucleic acid, peptide or other biological substance derived or isolated from such a mutant mouse, particularly a mutant, transgenic mouse which does not produce any functional NPC1L1, wherein the region of endogenous, chromosomal NPC1L1 deleted, in the mouse, corresponds to nucleotides 790-998 of the nucleotide sequence set forth in SEQ ID NO: 45.

[0179] The isolated cell can be isolated or derived, for example, from the duodenum, gall bladder, liver, small intestine or stomach of the mutant mouse. Further, the cell can be an enterocyte.

[0180] The *npc1l1*$^-$ mutant cells are useful, for example, for use in control experiments in screening assays (see *e.g.*, supra) since they lack any NPC1L1-dependent uptake or binding of

sterol, 5α-stanol or ezetimibe. The level of inhibition caused by a particular sample, in a screening assay, can be compared to that of an assay performed with the mutant cell. Ideally, though by no means necessarily, in a screening assay, for example, as described herein, the same amount of binding will be observed by a non-mutant cell or cell membrane, in the presence of an antagonist, as is observed in connection with a mutant *npc111* cell or cell membrane alone.

EXAMPLES

[0181] The following examples are provided to more clearly describe the present invention and should not be construed to limit the scope of the invention in any way.

Example 1: Cloning and Expression of Rat, Mouse and Human NPC1L1.

[00100] Rat *NPC*, mouse *NPC1L1* or human *NPC1L1* can all conveniently be amplified using polymerase chain reaction (PCR). In this approach, DNA from a rat, mouse or human cDNA library can be amplified using appropriate primers and standard PCR conditions. Design of primers and optimal amplification conditions constitute standard techniques which are commonly known in the art.

[0182] An amplified *NPC1L1* gene may conveniently be expressed, again, using methods which are commonly known in the art. For example, NPC1L1 may be inserted into a pET-based plasmid vector (Stratagene; La Joola, CA), downstream of the T7 RNA polymerase promoter. The plasmid may then be transformed into a T7 expression system (*e.g.*, BL21DE3 E.coli cells), grown in a liquid culture and induced (*e.g.*, by adding IPTG to the bacterial culture).

Example 2: Direct Binding Assay.

[0183] Membrane preparation: Caco2 cells transfected with an expression vector containing a polynucleotide encoding NPC1L1 (*e.g.*, SEQ ID NO: 2, 4 or 12) are harvested by incubating in 5 mM EDTA/phosphate-buffered saline followed by repeated pipeting. The cells are centrifuged 5 min at 1000 x g. The EDTA/PBS is decanted and an equal volume of ice-cold 50mM Tris-HCl, pH 7.5 is added and cells are broken up with a Polytron (PT10 tip, setting 5, 30 sec). Nuclei and unbroken cells are sedimented at 1000 x g for 10 min and then the supernatant is centrifuged at 50,000 x g for 10 min. The supernatant is decanted, the pellet is resuspended by Polytron, a sample is taken for protein assay (bicinchoninic acid, Pierce), and the tissue is again centrifuged at 50,000 x g. Pellets are stored frozen at –20°C.

[0184] Binding assay: For saturation binding, four concentrations of [^{3}H]-ezetimibe (15 Ci/mmol) are incubated without and with 10^{-5} M ezetimibe in triplicate with 50 μg of membrane protein in a total volume of 200 μl of 50 mM Tris-HCl, pH 7.5, for 30 min at 30°C. Samples are filtered on GF/B filters and washed three times with 2 ml of cold Tris buffer. Filters are dried in a microwave oven, impregnated with Meltilex wax scintillant, and counted at 45% efficiency. For competition binding assays, five concentrations of a sample are incubated in triplicate with 18 nM [^{3}H]-ezetimibe and 70 μg of membrane protein under the conditions described above. Curves are fit to the data with Prism (GraphPad Software) nonlinear least-squares curve-fitting program and K_i values are derived from IC_{50} values according to Cheng and Prusoff (Cheng, Y. C., *et al.*, (1973) Biochem. Pharmacol. 22: 3099-3108).

Example 3: SPA Assay.

[0185] For each well of a 96 well plate, a reaction mixture of 10 μg human, mouse or rat NPC1L1-CHO overexpressing membranes (Biosignal) and 200 μg/well YSi-WGA-SPA beads (Amersham) in 100 μl is prepared in NPC1L1 assay buffer (25 mM HEPES, pH 7.8, 2 mM CaCl2, 1mM MgCl2, 125 mM NaCl, 0.1% BSA). A 0.4 nM stock of ligand- [^{125}I]-ezetimibe- is prepared in the NPC1L1 assay buffer. The above solutions are added to a 96-well assay plate as follows: 50 μl NPC1L1 assay buffer, 100 μl of reaction mixture, 50 μl of ligand stock (final ligand concentration is 0.1 nM). The assay plates are shaken for 5 minutes on a plate shaker, then incubated for 8 hours before cpm/well are determined in Microbeta Trilux counter (PerkinElmer).

[0186] These assays will indicate that [^{125}I]-ezetimibe binds to the cell membranes expressing human, mouse or rat NPC1L1. Similar results will be obtained if the same experiment is performed with radiolabeled cholesterol (*e.g.*, ^{125}I-cholesterol).

Example 4: Cholesterol Uptake Assay.

[0187] CHO cells expressing either SR-B1 or three different clones of rat NPC1L1 or one clone of mouse NPC1L1 were starved overnight in cholesterol free media then dosed with [3H]-cholesterol in a mixed synthetic micelle emulsion for 4 min, 8 min, 12 min or 24 min in the absence or presence of 10 M ezetimibe. The cells were harvested and the lipids were organically extracted. The extracted lipids were spotted on thin-layer chromatography (TLC) plates and resolved within an organic vapor phase. The free cholesterol bands for each assay were isolated and counted in a scintillation counter.

[0188] The SR-B1 expressing cells exhibited an increase in [^{3}H]-cholesterol uptake as early as 4 min which was also inhibited by ezetimibe. The three rat clones and the one mouse clone appeared to give background levels of [^{3}H]-cholesterol uptake which was similar to that of the untransformed CHO cell.

[0189] These experiments will yield data demonstrating that CHO cells can perform mouse, rat and human NPC1L1-dependent uptake of [^{3}H]-cholesterol when more optimal experimental conditions are developed.

Example 5: Expression of Rat *NPC1L1* in Wistar Rat Tissue.

[0190] In these experiments, the expression of rat *NPC1L1* mRNA, in several rat tissues, was evaluated. The tissues evaluated were esophagus, stomach, duodenum, jejunum, ileum, proximal colon, distal colon, liver, pancreas, heart, aorta, spleen, lung, kidney, brain, muscle, testes, ovary, uterus, adrenal gland and thyroid gland. Total RNA samples were isolated from at least 3 male and 3 female animals and pooled. The samples were then subjected to real time quantitative PCR using Taqman analysis using standard dual-labeled fluorogenic oligonucleotide probes. Typical probe design incorporated a 5' reporter dye (*e.g.*, 6FAM (6-carboxyfluorescein) or VIC) and a 3' quenching dye (*e.g.*, TAMRA (6-carboxytetramethyl-rhodamine)).

rat *NPC1L1*:

[0191] Forward: TCTTCACCCTTGCTCTTTGC (SEQ ID NO: 14)

[0192] Reverse: AATGATGGAGAGTAGGTTGAGGAT (SEQ ID NO: 15)

[00101] Probe: [6FAM]TGCCCACCTTTGTTGTCTGCTACC[TAMRA] (SEQ ID NO: 16)

rat β-actin:

[0193] Forward: ATCGCTGACAGGATGCAGAAG (SEQ ID NO: 17)

[0194] Reverse: TCAGGAGGAGCAATGATCTTGA (SEQ ID NO: 18)

[00102] Probe: [VIC]AGATTACTGCCCTGGCTCCTAGCACCAT[TAMRA] (SEQ ID NO: 19)

[0195] PCR reactions were run in 96-well format with 25 µl reaction mixture in each well containing: Platinum SuperMix (12.5 µl), ROX Reference Dye (0.5 ul), 50 mM magnesium chloride (2 µl), cDNA from RT reaction (0.2 µl). Multiplex reactions contained gene specific primers at 200 nM each and FAM labeled probe at 100 nM and gene specific primers at 100 nM each and VIC labeled probe at 50 nM. Reactions were run with a standard 2-step cycling program, 95°C for 15 sec and 60°C for 1 min, for 40 cycles.

[0196] The highest levels of expression were observed in the duodenum, jejunum and ileum tissue. These data indicate that NPC1L1 plays a role in cholesterol absorption in the intestine.

Example 6: Expression of Mouse NPC1L1 in Mouse Tissue.

[0197] In these experiments, the expression of mouse *NPC1L1* mRNA, in several tissues, was evaluated. The tissues evaluated were adrenal gland, BM, brain, heart, islets of langerhans, LI, small intestine, kidney, liver, lung, MLN, PLN, muscle, ovary, pituitary gland, placenta, Peyers Patch, skin, spleen, stomach, testes, thymus, thyroid gland, uterus and trachea. Total RNA samples were isolate from at least 3 male and 3 female animals and pooled. The samples

were then subjected to real time quantitative PCR using Taqman analysis using the following primers and probes:

mouse *NPC1L1*:

[0198] Forward: ATCCTCATCCTGGGCTTTGC (SEQ ID NO: 20)

[0199] Reverse: GCAAGGTGATCAGGAGGTTGA (SEQ ID NO: 21)

[0200] Probe: [6FAM]CCCAGCTTATCCAGATTTTCTTCTTCCGC[TAMRA] (SEQ ID NO: 22)

[0201] The highest levels of expression were observed in the Peyer's Patch, small intestine, gall bladder and stomach tissue. These data are consistent with a cholesterol absorption role for NPC1L1 which takes place in the digestive system.

Example 7: Expression of Human NPC1L1 in Human Tissue.

[0202] In these experiments, the expression level of human NPC1L1 mRNA was evaluated in 2045 samples representing 46 normal tissues. Microarray-based gene expression analysis was performed on the Affymetrix HG-U95 GeneChip using a cRNA probe corresponding to base pairs 4192-5117 (SEQ ID NO: 43) in strict accordance to Affymetrix's established protocols. Gene Chips were scanned under low photo multiplier tube (PMT), and data were normalized using either Affymetrix MAS 4.0 or MAS 5.0 algorithms. In addition "spike ins" for most samples were used to construct a standard curve and obtain RNA concentration values according Gene Logic algorithms and procedures. A summary of these results are indicated, below, in Table 2.

Table 2. Expression level of *NPC1L1* mRNA in various human tissues.

Tissue	Present	Absent	Lower 25%	Median	Upper 75%	Tissue	Present	Absent	Lower 25%	Median	Upper 75%
Adipose	2 of 32	30 of 32	-2.45	1.16	12.23	Liver	32 of 34	2 of 34	325.74	427.77	540.1
Adrenal Gland	0 of 12	12 of 12	-23.54	-4.47	10.51	Lung	2 of 93	91 of 93	-3.47	11.03	22.34
Appendix	0 of 3	3 of 3	-8.02	-6.69	38.19	Lymph Node	0 of 11	11 of 11	-1.78	-0.19	1.34
Artery	0 of 3	3 of 3	-6.59	-4.67	9.68	Muscles	0 of 39	39 of 39	-21.57	8.25	26.73
Bladder	1 of 5	4 of 5	-22	-7.95	-1.99	Myometrium	8 of 106	98 of 106	-3.98	4.87	17.55
Bone	0 of 3	3 of 3	-1.64	3.3	19.53	Omentum	0 of 15	15 of 15	-14.25	-1.6	19.58
Breast	4 of 80	76 of 80	-4.07	3.13	14.67	Ovary	1 of 74	73 of 74	0.5	17.51	38.28
Cerebellum	0 of 5	5 of 5	-3.04	3.24	15.38	Pancreas	0 of 34	34 of 34	-87.08	-53.2	-24.14
Cervix	3 of 101	98 of 101	-7.56	-0.07	20.89	Placenta	0 of 5	5 of 5	-20.4	-3.44	18.91
Colon	9 of 151	142 of 151	-10.19	0.31	18.36	Prostate	0 of 32	32 of 32	1.08	15.56	27.24
Cortex Frontal Lobe	0 of 7	7 of 7	1.4	8.46	11.75	Rectum	1 of 43	42 of 43	-9.26	-1.49	9.8
Cortex Temporal Lobe	0 of 3	3 of 3	7.1	8.5	15.87	Right Atrium	4 of 169	165 of 169	-19.32	-6.58	7.72
Duodenum	59 of 61	2 of 61	519.23	827.43	1101.67	Right Ventricle	1 of 160	159 of 160	-24.01	-6.49	10.06
Endometrium	0 of 21	21 of 21	-14.43	-6.39	2.79	Skin	0 of 59	59 of 59	-12.68	1.5	22.77
Esophagus	1 of 27	26 of 27	-10.93	-4.97	12.48	Small Intestine	46 of 68	22 of 68	21.21	493.93	939.2
Falloplan Tube	3 of 51	48 of 51	5.02	13.24	26.77	Soft Tissues	1 of 6	5 of 6	-1.99	2.6	5.32
GallBladder	8 of 8	0 of 8	205.76	273.39	422.8	Spleen	0 of 31	31 of 31	-9.41	-0.31	9.5
Heart	0 of 3	3 of 3	3.33	11.19	11.66	Stomach	7 of 47	40 of 47	19.02	52.29	117.09
Hippocampus	0 of 5	5 of 5	8.25	9.11	19.83	Testis	0 of 5	5 of 5	-4.51	1.22	11.2
Kidney	4 of 86	82 of 86	-8.36	3.41	16.46	Thymus	1 of 71	70 of 71	-6.26	2.51	11.67
Larynx	0 of 4	4 of 4	-13.76	-0.81	8.54	Thyroid Gland	1 of 18	17 of 18	-12.22	2.84	17.86
Left Atrium	2 of 141	139 of 141	-18.9	-4.58	6.84	Uterus	0 of 58	58 of 58	-10.67	1.59	16.01
Left Ventricle	0 of 15	15 of 15	-21.19	-9.59	17.7	WBC	3 of 40	37 of 40	-16.45	-0.72	25.18

[0203] Shaded data corresponds to tissues wherein the highest levels of *NPC1L1* mRNA was detected. The "Present" column indicates the proportion of specified tissue samples evaluated wherein *NPC1L1* mRNA was detected. The "Absent" column indicates the proportion of specified tissue samples evaluated wherein *NPC1L1* RNA was not detected. The "lower 25%", "median" and "upper 75%" columns indicate statistical distribution of the relative NPC1L1 signal intensities observed for each set of tissue evaluated.

Example 8: Distribution of Rat *NPC1L1*, Rat *IBAT* or Rat *SR-B1* mRNA in Rat Small Intestine.

[0204] In these experiments, the distribution of rat *NPC1L1* mRNA along the proximal-distal axis of rat small intestines was evaluated. Intestines were isolated from five independent animals and divided into 10 sections of approximately equal length. Total RNA was isolated and analyzed, by real time quantitative PCR using Taqman analysis, for localized expression levels of rat *NPC1L1*, rat *IBAT* (ileal bile acid transporter) or rat *SR-B1* mRNA. The primers and probes used in the analysis were:

rat *NPC1L1*:

[0205] Forward: TCTTCACCCTTGCTCTTTGC (SEQ ID NO: 23)

[0206] Reverse: AATGATGGAGAGTAGGTTGAGGAT (SEQ ID NO: 24)

[0207] Probe: [6FAM]TGCCCACCTTTGTTGTCTGCTACC[TAMRA] (SEQ ID NO: 25)

rat Villin:

[0208] Forward: AGCACCTGTCCACTGAAGATTTC (SEQ ID NO: 26)

[0209] Reverse: TGGACGCTGAGCTTCAGTTCT (SEQ ID NO: 27)

[0210] Probe: [VIC]CTTCTCTGCGCTGCCTCGATGGAA[TAMRA] (SEQ ID NO: 28)

rat *SR*-B1:

[0211] Forward: AGTAAAAAGGGCTCGCAGGAT (SEQ ID NO: 29)

[0212] Reverse: GGCAGCTGGTGACATCAGAGA (SEQ ID NO: 30)

[0213] Probe: [6FAM]AGGAGGCCATGCAGGCCTACTCTGA[TAMRA] (SEQ ID NO: 31)

rat *IBAT*:

[0214] Forward: GAGTCCACGGTCAGTCCATGT (SEQ ID NO: 32)

[0215] Reverse: TTATGAACAACAATGCCAAGCAA (SEQ ID NO: 33)

[0216] Probe: [6FAM]AGTCCTTAGGTAGTGGCTTAGTCCCTGGAAGCTC[TAMRA] (SEQ ID NO: 34)

[0217] The mRNA expression levels of each animal intestinal section were analyzed separately, then the observed expression level was normalized to the observed level of villin mRNA in that intestinal section. The observed, normalized mRNA expression levels for each section where then averaged.

[0218] The expression level of *NPC1L1* and *SR-B1* were highest in the jejunum (sections 2-5) as compared to that of the more distal ileum sections. Since the jejunum is believed to be the site of cholesterol absorption, these data suggest such a role for rat NPC1L1. *IBAT* distribution favoring the ileum is well document and served as a control for the experiment.

Example 9: In situ Analysis of Rat *NPC1L1* mRNA in Rat Jejunum Tissue.

[0219] The localization of rat *NPC1L1* mRNA was characterized by *in situ* hybridization analysis of rat jejunum serial sections. The probes used in this analysis were:

[0220] T7-sense probe: GTAATACGACTCACTATAGGGCCCTGACGGTCCTTCCTGA GGGAATCTTCAC (SEQ ID NO: 35)

[0221] T7-antisense probe: GTAATACGACTCACTATAGGGCCTGGGAAGTTGGTCAT GGCCACTCCAGC (SEQ ID NO: 36)

[0222] The RNA probes were synthesized using T7 RNA polymerase amplification of a PCR amplified DNA fragment corresponding rat *NPC1L1* nucleotides 3318 to 3672 (SEQ ID NO 1). Sense and anti-sense digoxigenin–UTP labeled cRNA probes were generated from the T7 promoter using the DIG RNA Labeling Kit following the manufacturer's instructions. Serial cryosections rat jejunum were hybridized with the sense and antiisense probes. Digoxigenin labeling was detected with the DIG Nucleic Acid Detection Kit based on previous methods. A positive signal is characterized by the deposition of a red reaction product at the site of hybridization.

[0223] The anti-sense probe showed strong staining of epithelium along the crypt-villus axis under low magnification (40X). The observed rat *NPC1L1* mRNA expression levels may have been somewhat greater in the crypts than in the villus tips. Under high magnification (200X), staining was observed in the enterocytes but not in the goblet cells. A lack of staining observed with the sense probe (control) confirmed the high specificity of the *NPC1L1* anti-sense signal. These data provided further evidence of the role of rat NPC1L1 in intestinal cholesterol absorption.

Example 10: FACS Analysis of Fluorescently Labeled Ezetimibe Binding to Transiently Transfected CHO Cells.

[0224] In these experiments, the ability of BODIPY-labeled ezetimibe (Altmann, *et al.*, (2002) Biochim. Biophys. Acta 1580(1): 77-93) to bind to *NPC1L1* and *SR-B1* was evaluated. "BODIPY" is a fluorescent group which was used to detect the BODIPY-ezetimibe. Chinese hamster ovary (CHO) cells were transiently transfected with rat *NPC1L1* DNA (rNPC1L1/CHO), mouse *NPC1L1* DNA (mNPC1L1/CHO), mouse *SR-B1* DNA (mSRBI/CHO) or *EGFP* DNA (EGFP/CHO). EGFP is enhanced green fluorescent protein which was used as a positive control. The transfected CHO cells or untransfected CHO cells were then stained with 100 nM BODIPY-labeled ezetimibe and analyzed by FACS. Control experiments were also performed wherein the cells were not labeled with the BODIPY-ezetimibe and wherein untransfected CHO cells were labeled with the BODIPY-ezetimibe.

[0225] No staining was observed in the untransfected CHO, rNPC1L1/CHO or mNPC1L1/CHO cells. Fluorescence was detected in the positive-control EGFP/CHO cells. Staining was also detected in the mouse SR-B1/CHO cells. These data show that, under the conditions tested, BODIPY-ezetimibe is capable of binding to SR-B1 and that such binding is not ablated by the presence of the fluorescent BODIPY group. When more optimal conditions are determined, BODIPY-ezetimibe will be shown to label the rNPC1L1/CHO and mNPC1L1/CHO cells.

Example 11: FACS Analysis of Transiently Transfected CHO Cells Labeled with Anti-FLAG Antibody M2.

[0226] In these experiments, the expression of FLAG-tagged NPC1L1 on CHO cells was evaluated. CHO cells were transiently transfected with mouse *NPC1L1* DNA, rat *NPC1L1*

DNA, FLAG- rat *NPC1L1* DNA or FLAG- mouse *NPC1L1* DNA. The 8 amino acid FLAG tag used was DYKDDDDK (SEQ ID NO: 37) which was inserted on the amino-terminal extracellular loop just past the secretion signal sequence. The cells were incubated with commercially available anti-FLAG monoclonal mouse antibody M2 followed by a BODIPY-tagged anti-mouse secondary antibody. The treated cells were then analyzed by FACS.

[0227] The M2 antibody stained the CHO cells transfected with FLAG-rat *NPC1L1* DNA and with FLAG–mouse NPC1L1. No staining was observed in the CHO cells transfected with mouse *NPC1L1* DNA and with rat *NPC1L1* DNA. These data showed that rat NPC1L1 and mouse NPC1L1 possess no significant, inherent fluorescence and are not bound by the anti-FLAG antibody. The observed, FLAG-dependent labeling of the cells indicated that the FLAG-mouse NPC1L1 and FLAG-rat NPC1L1 proteins are localized at the cell membrane of the CHO cells.

Example 12: FACS Analysis of FLAG-rat NPC1L1-EGFP Chimera in Transiently Transfected CHO Cells.

[0228] In these experiments, the surface and cytoplasmic localization of rat NPC1L1 in CHO cells was evaluated. CHO cells were transiently transfected with FLAG- rat *NPC1L1* DNA or with FLAG-rat *NPC1L1-EGFP* DNA. In these fusions, the FLAG tag is at amino-terminus of rat NPC1L1 and EGFP fusion is at the carboxy-terminus of rat NPC1L1. The cells were then stained with the M2 anti-FLAG mouse (primary) antibody followed by secondary staining with a BODIPY-labeled anti-mouse antibody. In control experiments, cells were stained with only the

secondary antibody and not with the primary antibody (M2). The stained cells were then analyzed by FACS.

[0229] In a control experiment, FLAG-rat NPC1L1 transfected cells were stained with BODIPY anti-mouse secondary antibody but not with the primary antibody. The data demonstrated that the secondary, anti-mouse antibody possessed no significant specificity for FLAG-rat NPC1L1 and that the FLAG-rat NPC1L1, itself, possesses no significant fluorescence.

[0230] In another control experiment, unlabeled FLAG-rat NPC1L1-EGFP cells were FACS analyzed. In these experiments, autofluorescence of the enhanced green fluorescent protein (EGFP) was detected.

[0231] FLAG-rat NPC1L1 cells were stained with anti-FLAG mouse antibody M2 and with the BODIPY-labeled anti-mouse secondary antibody and FACS analyzed. The data from this analysis showed that the cells were labeled with the secondary, BODIPY-labeled antibody which indicated expression of the FLAG-rat NPC1L1 protein on the surface of the CHO cells.

[0232] FLAG-rat NPC1L1-EGFP cells were stained with anti-FLAG mouse antibody M2 and with the BODIPY-labeled anti-mouse secondary antibody and FACS analyzed. The data from this analysis showed that both markers (BODIPY and EGFP) were present indicating surface expression of the chimeric protein. The data also indicated that a portion of the protein was located within the cells and may be associated with transport vesicles. These data supported a role for rat NPC1L1 in vesicular transport of cholesterol or protein expressed in subcellular organelles such as the rough endoplasmic reticulum.

Example 13: FACS Analysis and Fluorescent Microscopy of FLAG-rat NPC1L1-EGFP Chimera in a Cloned CHO Cell Line.

[0233] In these experiments, the cellular localization of rat NPC1L1 was evaluated by FACS analysis and by immunohistochemistry. CHO cells were transfected with FLAG-rat *NPC1L1-EGFP* DNA and stained with anti-FLAG mouse antibody M2 and then with a BODIPY-labeled anti-mouse secondary antibody. In the fusion, the FLAG tag is at the amino-terminus of rat NPC1L1 and the enhanced green fluorescent protein (EGFP) tag is located at the carboxy-terminus of the rat NPC1L1. The stained cells were then analyzed by FACS and by fluorescence microscopy.

[0234] Cells transfected with FLAG–rat *NPC1L1-EGFP* DNA were stained with the anti-FLAG mouse antibody M2 and then with the BODIPY-labeled anti-mouse secondary antibody. FACS analysis of the cells detected both markers indicating surface expression of the chimeric protein.

[0235] FLAG–rat NPC1L1-EGFP transfected cells were analyzed by fluorescent microscopy at 63X magnification. Fluorescent microscopic analysis of the cells indicated non-nuclear staining with significant perinuclear organelle staining. Resolution of the image could not confirm the presence of vesicular associated protein. These data indicated that the fusion protein was expressed on the cell membrane of CHO cells.

Example 14: Generation of Polyclonal Anti-rat NPC1L1 Rabbit Antibodies.

[0236] Synthetic peptides (SEQ ID NO: 39-42) containing an amino- or carboxy-terminal cysteine residue were coupled to keyhole limpet hemocyanin (KLH) carrier protein through a

disulfide linkage and used as antigen to raise polyclonal antiserum in New Zealand white rabbits (range 3-9 months in age). The KLH-peptide was emulsified by mixing with an equal volume of Freund's Adjuvant, and injected into three subcutaneous dorsal sites. Prior to the 16 week immunization schedule a pre-immune sera sample was collected which was followed by a primary injection of 0.25 mg KLH-peptide and 3 scheduled booster injections of 0.1 mg KLH-peptide. Animals were bled from the auricular artery and the blood was allowed to clot and the serum was then collected by centrifugation

[0237] The anti-peptide antibody titer was determined with an enzyme linked immunosorbent assay (ELISA) with free peptide bound in solid phase (1μg/well). Results are expressed as the reciprocal of the serum dilution that resulted in an OD_{450} of 0.2. Detection was obtained using the biotinylated anti-rabbit IgG, horse radish peroxidase–streptavidin (HRP-SA) conjugate, and ABTS.

Example 15: FACS Analysis of Rat NPC1L1 Expression in CHO Cells Transiently Transfected with Rat NPC1L1 DNA Using Rabbit Anti-rat NPC1L1 Antisera.

[00103] In these experiments, the expression of rat NPC1L1 on the surface of CHO cells was evaluated. CHO cells were transfected with rat *NPC1L1* DNA, then incubated with either rabbit preimmune serum or with 10 week anti-rat NPC1L1 serum described, above, in Example 14 (i.e., A0715, A0716, A0867 or A0868). Cells labeled with primary antisera were then stained with a BODIPY-modified anti-rabbit secondary antibody followed by FACS analysis.

[0238] No antibody surface labeling was observed for any of the pre-immune sera samples. Specific cell surface labeling of rat NPC1L1 transfected cells was observed for both A0715 and

A0868. Antisera A0716 and A0867 did not recognize rat NPC1L1 surface expression in this assay format. This indicates that the native, unfused rat NPC1L1 protein is expressed in the CHO cells and localized to the CHO cell membranes. Cell surface expression of NPC1L1 is consistent with a role in intestinal cholesterol absorption.

Example 16: FACS Analysis of CHO Cells Transiently Transfected with FLAG-Mouse NPC1L1 DNA or FLAG-rat NPC1L1 DNA or Untransfected CHO Cells Using Rabbit Anti-rat NPC1L1 Antisera.

[0239] In these experiments, the expression of FLAG-mouse NPC1L1 and FLAG-rat NPC1L1 in CHO cells was evaluated. CHO cells were transiently transfected with FLAG-mouse *NPC1L1* DNA or with FLAG-rat *NPC1L1* DNA. The FLAG-mouse NPC1L1 and FLAG-rat NPC1L1 transfected cells were labeled with either A0801, A0802, A0715 or A0868 sera (see Example 14) or with anti-FLAG antibody, M2. The labeled cells were then stained with BODIPY-labeled anti-rabbit secondary antibody and FACS analyzed. The untransfected CHO cells were analyzed in the same manner as the transfected cell lines.

[0240] Positive staining of the untransfected CHO cells was not observed for any of the antisera tested. Serum A0801-dependent labeling of FLAG-rat NPC1L1 transfected cells was observed but such labeling of FLAG-mouse NPC1L1 transfected cells was not observed. Serum A0802-dependent labeling of FLAG-mouse NPC1L1 or FLAG-rat NPC1L1 transfected cells was not observed. Strong serum A0715-dependent labeling of FLAG-rat NPC1L1 transfected cells was observed and weak serum A0715-dependent labeling of FLAG-mouse NPC1L1 transfected cells was observed. Weak serum A0868-dependent labeling of rat NPC1L1 and mouse NPC1L1

transfected cells was observed. Strong Anti-FLAG M2 antibody-dependent labeling of FLAG-rat NPC1L1 and FLAG-mouse NPC1L1 transfected cells was observed. The strong M2 staining is likely to be due to the fact that M2 is an affinity-purified, monoclonal antibody of known concentration. In contrast, the respective antisera are polyclonal, unpurified and contain an uncertain concentration of anti-rat NPC1L1 antibody. These date provide further evidence that the FLAG-mouse NPC1L1 and FLAG-rat NPC1L1 proteins are expressed in CHO cells and localized to the CHO cell membranes. Cell surface expression of NPC1L1 is consistent with a role in intestinal cholesterol absorption.

Example 17: Immunohistochemical Analysis of Rat Jejunum Tissue with Rabbit Anti-rat NPC1L1 Antisera A0715.

[0241] In these experiments, the localization of rat NPC1L1 in rat jejunum was analyzed by immunohistochemistry. Rat jejunum was removed, immediately embedded in O.C.T. compound and frozen in liquid nitrogen. Sections (6μm) were cut with a cryostat microtome and mounted on glass slides. Sections were air dried at room temperature and then fixed in Bouin's fixative. Streptavidin-biotin-peroxidase immunostaining was carried out using Histostain-SP kit. Endogenous tissue peroxidase activity was blocked with a 10 minute incubation in 3% H_2O_2 in methanol, and nonspecific antibody binding was minimized by a 45 minute incubation in 10% nonimmune rabbit serum. Sections were incubated with a rabbit anti-rat NPC1L1 antisera A0715 or A0868 at a 1: 500 dilution at 4°C, followed by incubation with biotinylated goat anti-rabbit IgG and with streptavidin-peroxidase. Subsequently, the sections were developed in an aminoethyl carbazole (AEC)-H2O2 staining system and counterstained with hematoxylin and examined by microscopy. A positive reaction using this protocol is characterized by the

deposition of a red reaction product at the site of the antigen-antibody reaction. Nuclei appeared blue from the hematoxylin counterstain. Controls were performed simultaneously on the neighboring sections from the same tissue block. Control procedures consisted of the following: (1) substitute the primary antibody with the pre-immune serum, (2) substitute the primary antibody with the non-immune rabbit serum, (3) substitute the primary antibody with PBS, (4) substitute the second antibody with PBS.

[0242] The example shows tissue stained with anti-rat NPC1L1 sera A0715 or with the preimmune sera analyzed at low magnification (40X) and at high magnification (200X). The A0715-stained tissue, at low magnification, showed positive, strong staining of the villi epithelial layer (enterocytes). The A0715-stained tissue at high magnification showed positive, strong staining of the enterocyte apical membranes. No staining was observed in tissue treated only with preimmune sera. Similar results were obtained with sera A0868. These data indicate that rat NPC1L1 is expressed in rat jejunum which is consistent with a role in intestinal cholesterol absorption.

Example 18: Labeled Cholesterol Uptake Assay.

[0243] In this example, the ability of CHO cells stably transfected with rat NPC1L1 to take up labeled cholesterol was evaluated. In these assays, cholesterol uptake, at a single concentration, was evaluated in a pulse-chase experiment. The data generated in these experiments are set forth, below, in Table 3.

[0244] Cells:

A. CHO cells stably transfected with rat *NPC1L1* cDNA

B. CHO background (no transfection)

[0245] Cells were seeded at 500,000 cells/ well (mL) in 12-well plates.

Procedure:

[0246] All reagents and culture plates were maintained at 37°C unless otherwise noted.

[0247] **Starve.** The maintenance media (F12 HAMS, 1%Pen/Strep, 10%FCS) was removed and the cells were rinsed with serum-free HAMS media. The serum-free media was then replaced with 1 mL "starve" media (F12 HAMS, Pen/Strep, 5% lipoprotein deficient serum (LPDS).

[0248] One plate of each cell line was starved overnight. The remaining 2 plates were designated "No Starve" (see below).

[0249] **Pre-Incubation.** Media was removed from all plates, rinsed with serum-free HAMS and replaced with starve media for 30 minutes.

[0250] **^{3}H-Cholesterol Pulse.** The following was added directly to each well.

[0251] 0.5μCi ^{3}H-cholesterol (~1.1 X 10^{6} dpm/well) in 50μl of a mixed bile salt micelle.

4.8mM sodium taurocholate (2.581mg/mL)

0.6 mM sodium oleate (0.183mg/mL)

0.25 mM cholesterol (0.1 mg/mL)

Dispersed in "starve" media by ultrasonic vibration

Final media cholesterol concentration = 5μg/mL

[0252] Labeled cholesterol pulse time points were 0, 4, 12 and 24 minutes. Triplicate wells for each treatment were prepared.

[0253] **Wash.** At the designated times, media was aspirated and the cells were washed once with Hobbs Buffer A (50mM Tris, 0.9% NaCl, 0.2% BSA, pH 7.4) and once with Hobbs Buffer B (50mM Tris, 0.9% NaCl, pH 7.4 (no BSA)) at 37oC.

[0254] **Processing/Analysis.** Cells were digested overnight with 0.2N NaOH, 2mL/well at room temperature. One 1.5 mL aliquot was removed from each well, neutralized & counted for radioactivity by scintillation counting. Two additional 50µl aliquots from all wells are assayed for total protein by the Pierce micro BCA method. The quantity of labeled cholesterol observed in the cells was normalized by the quantity of protein in the cells.

Table 3. Uptake of 3H-cholesterol by CHO cells transfected with rat *NPC1L1* or mouse *SR-B1* or untransfected CHO cells.

Time, min After ^{3}H-Cholesterol	Total Cholesterol, dpm protein ± sem				Total Cholesterol, dpm/mg protein ± sem			
	NPC1L1		CHO		NPC1L1		CHO	
				No Starve				
0	2067	±46	4568	±1937	10754	±166	22881	±9230
4	2619	±130	2868	±193	15366	±938	15636	±1471
12	2868	±193	4459	±170	15636	±1471	24622	±966
24	7010	±89	7204	±173	41129	±685	39361	±1207
				Starve				
0	1937	±273	2440	±299	10909	±1847	12429	±1673
4	3023	±308	2759	±105	17278	±1650	14307	±781
12	2759	±105	4857	±186	14307	±781	26270	±1473
24	6966	±72	7344	±65	39196	±174	38381	±161

dpm=disintegrations per minute
sem=standard error of the mean

Example 19: Effect of Ezetimibe on Cholesterol Uptake.

[0255] The effect of ezetimibe on the ability of CHO cells stably transfected with mouse or rat *NPC1L1* or mouse *SR-B1* to take up ^{3}H-labeled cholesterol was evaluated in pulse-chase experiments. One cDNA clone of mouse *NPC1L1* (C7) and three clones of rat *NPC1L1* (C7, C17 and C21) were evaluated. The ability of CHO cells stably transfected with mouse *SR-B1*, mouse *NPC1L1* and rat *NPC1L1* to take up labeled cholesterol, in the absence of ezetimibe, was

also evaluated in the pulse-chase experiments. Data generated in these experiments are set forth, below, in Tables 4 and 5. Additionally, the quantity of total cholesterol taken up by transfected and untransfected CHO cells in the presence of four different unlabeled cholesterol concentrations was also evaluated. The data from these experiments is set forth, below, in Table 6.

[0256] Cells:

A. CHO cells stably transfected with rat or mouse *NPC1L1* cDNA

B. CHO background (no transfection)

C. *SR-B1* transfected CHO cells

Cells seeded at 500,000 cells / well (mL) in 12-well plates.

Procedure:

[0257] All reagents and culture plates were maintained at 37°C unless otherwise noted.

[0258] **Starve.** The maintenance media (F12 HAMS, 1%Pen/Strep, 10%FCS) was removed and the cells were rinsed with serum-free HAMS media. The serum-free media was then replaced with 1 mL "starve" media (F12 HAMS, Pen/Strep, 5% lipoprotein deficient serum (LPDS). The cells were then starved overnight.

[0259] **Pre-Incubation/ pre-dose.** Media was removed from all plates and replaced with fresh starve media and preincubated for 30 minutes. Half of the wells received media containing ezetimibe (stock soln in EtOH; final conc. = 10μM).

[0260] **^{3}H-Cholesterol Pulse.** The following was added directly to each well:

0.5μCi ^{3}H-cholesterol (~1.1 X 106 dpm/well) in 50μl of a mixed bile salt micelle

4.8mM sodium taurocholate (2.581mg/mL)

0.6 mM sodium oleate (0.183mg/mL)

0.25 mM cholesterol (0.1 mg/mL)

Dispersed in "starve" media by ultrasonic vibration

Final media cholesterol concentration = 5μg/mL

[0261] Labeled cholesterol pulse time points were 4, 12, 24 minutes and 4 hours. Triplicate wells were prepared for each treatment.

[0262] **Wash.** At designated times, media was aspirated and cells were washed once with Hobbs Buffer A (50mM Tris, 0.9% NaCl, 0.2% bovine serum albumin (BSA), pH 7.4) and once with Hobbs Buffer B (50mM Tris, 0.9% NaCl, pH 7.4 (no BSA)) at 37oC.

Processing/Analysis.

[0263] A. 4, 12, 24 minute time points: Cells were digested overnight with 0.2N NaOH, 2mL/well, room temperature. One 1.5 mL aliquot was removed from each well, neutralized & counted for radioactivity by scintillation counting.

[0264] B. 4 hour time point: The digested cells were analyzed by thin-layer chromatography to determine the content of cholesterol ester in the cells.

[0265] Extracts were spotted onto TLC plates and run for 30 minutes in 2 ml hexane: isopropanol (3: 2) mobile phase for 30 minutes, followed by a second run in 1ml hexane: isopropanol (3: 2) mobile phase for 15 minutes.

[0266] C. Protein determination of cell extracts. Plates containing a sample of the cell extracts were placed on orbital shaker at 120 rpm for indicated times and then extracts are pooled into 12 X 75 tubes. Plates were dried and NaOH (2ml/well) added. The protein content of the samples were then determined. Two additional 50µl aliquots from all wells were assayed for total protein by the Pierce micro BCA method. The quantity of labeled cholesterol observed in the cells was normalized to the quantity of protein in the cells.

Table 4. Total Cholesterol in Transfected CHO Cells in the Presence and Absence of Ezetimibe.

	Total Cholesterol, dpm ± sem				Total Cholesterol, dpm/mg protein ± sem			
	Vehicle		EZ (10 μM)		Vehicle		EZ (10μM)	
Clones:	4 Min Pulse							
CHO Control	3413	±417	3222	±26	33443	±4070	31881	±483
SR-BI	14207	±51	10968	±821	118242	±1261	92474	±2902
mNPC1L1(C7)	4043	±419	4569	±222	30169	±3242	30916	±1137
rNPC1L1(C21)	3283	±288	3769	±147	23728	±2111	27098	±689
rNPC1L1(C17)	3188	±232	3676	±134	24000	±832	28675	±527
rNPC1L1(C7)	1825	±806	3268	±121	15069	±6794	27285	±968
	12 Min Pulse							
CHO Control	4710	±246	4532	±165	44208	±2702	43391	±1197
SR-BI	16970	±763	12349	±298	140105	±6523	98956	±4447
mNPC1L1(C7)	6316	±85	6120	±755	45133	±342	41712	±4054
rNPC1L1(C21)	5340	±12	4703	±231	40018	±1181	33985	±1928
rNPC1L1(C17)	4831	±431	4579	±257	37378	±3461	34063	±1619
rNPC1L1(C7)	4726	±272	4664	±63	39100	±2350	38581	±784
	24 Min Pulse							
CHO Control	7367	±232	6678	±215	65843	±1281	61764	±2131
SR-BI	39166	±2152	23558	±1310	324126	±11848	198725	±11713
mNPC1L1(C7)	10616	±121	9749	±482	77222	±1040	74041	±3670
rNPC1L1(C21)	9940	±587	8760	±293	76356	±9618	66165	±2181
rNPC1L1(C17)	8728	±721	8192	±237	70509	±5189	62279	±4352
rNPC1L1(C7)	8537	±148	7829	±204	72134	±1305	63482	±368

EZ = ezetimibe

Table 5. Cholesterol Ester in CHO cells in the Presence or Absence of Ezetimibe.

Table 5. Cholesterol Ester in CHO cells in the Presence or Absence of Ezetimibe.

	Cholesteryl Ester, dpm ± sem				Cholesteryl Ester, dpm/mg protein ± sem			
	Vehicle		EZ (10 μM)		Vehicle		EZ (10μM)	
Clones:	4 Hour Pulse							
CHO Control	652	±13	208	±9	5647	±55	1902	±87
SR-BI	47608	±1292	9305	±401	391067	±14391	72782	±3181
mNPC1L1(C7)	732	±127	453	±118	4994	±827	3057	±776
rNPC1L1(C21)	2667	±90	454	±33	18655	±1032	3193	±265
rNPC1L1(C17)	751	±74	202	±10	5379	±481	1510	±62
rNPC1L1(C7)	462	±25	191	±54	3597	±193	1496	±403

	Free Cholesterol, dpm ± sem				Free Cholesterol, dpm/mg protein ± sem			
	Vehicle		EZ (10 μM)		Vehicle		EZ (10μM)	
	4 Hour Pulse							
CHO Control	61612	±1227	56792	±568	533876	±17770	519607	±16203
SR-BI	214678	±4241	194519	±474	1762873	±46607	1521341	±4185
mNPC1L1(C7)	79628	±793	77516	±1910	544661	±1269	523803	±10386
rNPC1L1(C21)	71352	±1343	69106	±711	498016	±8171	485460	±4410
rNPC1L1(C17)	78956	±3782	71646	±446	566456	±29204	536651	±7146
rNPC1L1(C7)	75348	±2093	70628	±212	586127	±13932	556855	±7481

EZ =ezetimibe

Table 6. Uptake of labeled cholesterol in the presence of increasing amounts of unlabeled cholesterol.

Cold Cholesterol	Total Cholesterol, dpm ± sem				Total Cholesterol, dpm/mg protein ± sem			
	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)
				24 Min Pulse				
3 µg/mL	12271 ±430	49603 ±2428	14250 ±1628	10656 ±1233	108936 ±5413	541562 ±13785	140764 ±14433	94945 ±12916
10 µg/mL	16282 ±2438	79967 ±8151	25465 ±3037	13225 ±4556	151283 ±23345	880224 ±82254	250985 ±27481	123433 ±34092
30 µg/mL	14758 ±1607	71925 ±3863	19001 ±1530	13218 ±1149	135109 ±12106	796236 ±18952	180436 ±12112	111522 ±6941
100 µg/mL	16458 ±1614	58185 ±4548	15973 ±1665	11560 ±1132	149559 ±17977	630143 ±3718	147717 ±8261	101328 ±7191
	Cholesteryl Ester, dpm ± sem				Cholesteryl Ester, dpm/mg protein ± sem			
	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)
				4 Hour Pulse				
3 µg/mL	2737 ±114	39596 ±1241	1561 ±1	4015 ±47	22050 ±978	382641 ±5955	13684 ±217	32020 ±641
10 µg/mL	1646 ±76	17292 ±362	998 ±36	1866 ±33	13323 ±606	157914 ±3400	8917 ±467	14849 ±127
30 µg/mL	970 ±46	6642 ±153	537 ±82	970 ±9	7627 ±325	63547 ±1760	4885 ±748	7741 ±100
100 µg/mL	895 ±156	4777 ±27	405 ±7	777 ±16	7135 ±1230	45088 ±1526	3663 ±68	6005 ±198
	Free Cholesterol, dpm ± sem				Free Cholesterol, dpm/mg protein ± sem			
	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)
				4 Hour Pulse				
3 µg/mL	89013 ±3724	211783 ±3268	104343 ±2112	92244 ±987	717308 ±34130	2047695 ±16213	914107 ±5869	735498 ±11209
10 µg/mL	136396 ±8566	278216 ±10901	196173 ±4721	125144 ±877	1105118 ±76074	2540130 ±92471	1753072 ±86578	996824 ±27850
30 µg/mL	131745 ±2922	224429 ±2556	149172 ±19689	117143 ±4976	1036195 ±21142	2149315 ±78068	1357136 ±180264	934772 ±43202
100 µg/mL	79336 ±4011	231470 ±4221	114599 ±2803	93538 ±1588	632965 ±29756	2182022 ±36793	1035979 ±30329	723225 ±21694
	Cholesteryl Ester, dpm ± sem				Cholesteryl Ester, dpm/mg protein ± sem			
	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)
				24 Hour Pulse				
3 µg/mL	57373 ±2704	162296 ±1644	22986 ±940	59377 ±953	357629 ±14639	1248900 ±18565	160328 ±6565	401315 ±5557
10 µg/mL	33730 ±1296	112815 ±373	14836 ±552	31797 ±525	215004 ±5942	830231 ±12764	98594 ±4205	200451 ±5239
30 µg/mL	19193 ±100	58668 ±1413	8878 ±355	18963 ±380	122071 ±1271	446581 ±3472	59091 ±2697	119728 ±2131
100 µg/mL	16761 ±398	31280 ±1270	8784 ±946	14933 ±311	103235 ±1739	272796 ±13392	60670 ±4597	96215 ±1023
	Free Cholesterol, dpm ± sem				Free Cholesterol, dpm/mg protein ± sem			
	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)
				24 Hour Pulse				
3 µg/mL	248985 ±4207	357819 ±4519	285610 ±5187	227244 ±1016	1552637 ±18954	2752957 ±24984	1993256 ±56968	1536023 ±10304
10 µg/mL	231208 ±8927	269822 ±5872	311777 ±8227	231666 ±6198	1477414 ±85954	1984473 ±18420	2069980 ±25517	1461157 ±58517
30 µg/mL	203566 ±6008	225273 ±5932	279604 ±6612	209372 ±3386	1294878 ±41819	1716066 ±52581	1859476 ±29507	1321730 ±5452
100 µg/mL	178424 ±2379	167082 ±2211	229832 ±4199	182678 ±7709	1099648 ±25160	1455799 ±9885	1599244 ±76938	1177546 ±51191

Example 20: Labeled Cholesterol Uptake Assay.

[0267] In this example, the ability of CHO cells transiently transfected with rat *NPC1L1* or mouse *SR-B1* to take up labeled cholesterol was evaluated. Also evaluated was the ability of rat *NPC1L1* to potentiate the ability of CHO cells transfected with mouse *SR-B1* to take up labeled cholesterol. In these assays, cholesterol uptake, at a single concentration, was evaluated in pulse-chase experiments. The data generated in these experiments are set forth, below, in Table 7.

[0268] Cells:

A. CHO background cells (mock transfection).

B. CHO cells transiently transfected with mouse SR-B1.

C. CHO transiently transfected with rat *NPC1L1* cDNAs (n=8 clones).

[0269] Transiently transfected cells were seeded at 300,000 cells / well (mL) in 12-well plates.

Procedure:

[0270] All reagents and culture plates were maintained at 37°C unless otherwise noted.

[0271] Starve. The maintenance media (F12 HAMS, 1%Pen/Strep, 10%FCS) was removed from the cells and replaced with 1 mL "starve" media (F12 HAMS, Pen/Strep, 5% lipoprotein deficient serum (LPDS). Cells were starved for 1 hour.

[0272] ^{3}H-Cholesterol Pulse. The following was added directly to each well.

[0273] 0.5μCi ^{3}H-cholesterol (~1.1 X 106 dpm/well) in 50μl of a mixed bile salt micelle.

4.8mM sodium taurocholate (2.581mg/mL)

0.6 mM sodium oleate (0.183mg/mL)

0.25 mM cholesterol (0.1 mg/mL)

Dispersed in "starve" media by ultrasonic vibration

Final media cholesterol concentration = 5μg/mL

[0274] Labeled cholesterol pulse time points were 24 Min and 4 hours. Triplicate wells for each treatment.

[0275] Wash. At the designated times, media was aspirated and cells were washed once with Hobbs Buffer A (50mM Tris, 0.9% NaCl, 0.2% BSA, pH 7.4) and once with Hobbs Buffer B (50mM Tris, 0.9% NaCl, pH 7.4 (no BSA)) at 37oC.

Processing/Analysis.

[0276] A. 24 minute time point: Cells were digested overnight with 0.2N NaOH, 2mL/well at room temp. One 1.5 mL aliquot was removed from each well, neutralized & counted for radioactivity by scintillation counting.

[0277] B. 4 hour time point: The digested cells were analyzed by thin-layer chromatography to determine the content of cholesterol ester in the cells.

[0278] The extracts were spotted onto thin layer chromatography plates and run in 2 ml hexane: isopropanol (3: 2) containing mobile phase for 30 minutes, followed by a second run in 1ml hexane: isopropanol (3: 2) containing mobile phase for 15min.

[0279] C. Protein determination of cell extracts: Plates containing a sample of the cell extracts were placed on orbital shaker at 120 rpm for indicated times and then extracts are

pooled into 12X75 tubes. Plates were dried and NaOH (2ml/well) added. The protein content of the samples were then determined. Two additional 50µl aliquots from all wells were assayed for total protein by the Pierce micro BCA method. The quantity of labeled cholesterol observed in the cells was normalized to the quantity of protein in the cells.

Table 7. Labeled cholesterol uptake in transiently transfected CHO cells.

Transfection	Total Cholesterol, ± sem dpm	Total Cholesterol, ± sem dpm/mg protein
	24 Min Pulse	
CHO Control (mock)	4721 ±436	49024 ±4328
SR-BI(Transient)	5842 ±82	59445 ±1099
NPC1L1 (Transient)	4092 ±377	47026 ±2658
SR-BI/NPC1L1 (trans)	3833 ±158	52132 ±3071
	Cholesteryl Ester, ± sem dpm	**Cholesteryl Ester, ± sem dpm/mg protein**
	4 Hour Pulse	
CHO Control (mock)	2132 ±40	20497 ±640
SR-BI(Transient)	5918 ±237	51812 ±1417
NPC1L1 (Transient)	1944 ±93	19788 ±642
SR-BI/NPC1L1 (trans)	4747 ±39	58603 ±1156
	Free Cholesterol, ± sem dpm	**Free Cholesterol, ± sem dpm/mg protein**
	4 Hour Pulse	
CHO Control (mock)	45729 ±328	439346 ±5389
SR-BI(Transient)	50820 ±2369	444551 ±9785
NPC1L1 (Transient)	39913 ±1211	406615 ±6820
SR-BI/NPC1L1 (trans)	37269 ±1225	459509 ±6195

Example 21: Expression of rat, mouse and human NPC1L1.

[0280] In this example, *NPC1L1* was introduced into cells and expressed. Species specific NPC1L1 expression constructs were cloned into the plasmid pCDNA3 using clone specific PCR

primers to generate the ORF flanked by appropriate restriction sites compatible with the polylinker of the vector. For all three species of NPC1L1, small intestine total tissue RNA was used as a template for reverse transcriptase-polymerase chain reaction (RT-PCR) using oligo dT as the template primer. The rat *NPC1L1* was cloned as an EcoRI fragment, human *NPC1L1* was cloned as a XbaI/NotI fragment and mouse *NPC1L1* was cloned as an EcoRI fragment. Forward and reverse strand sequencing of each clone was performed to confirm sequence integrity. Standard transient transfection procedures were used with CHO cells. In a 6-well plate CHO cells were plated 1 day before transfection at a plating density of 2 X 10^5 cells/well. The following day, cells were incubated with 2 μg plasmid DNA and 6 μL Lipofectamine for 5 hours followed a fresh media change. Forty-eight hours later, cells were analyzed for NPC1L1 expression using anti-NPC1L1 antisera by either FACS or western blot. To establish stable long term cell lines expressing NPC1L1, transfected CHO cells were selected in the presence of geneticin (G418, 0.8 mg/ml) as recommended by the manufacturer (Life Technologies). Following one month of selection in culture, the cell population was stained with anti-NPC1L1 antisera and sorted by FACS. Individual positive staining cells were cloned after isolation by limiting dilution and then maintained in selective media containing geneticin (0.5 mg/ ml).

[0281] Other cell types less susceptible to transfection procedures have been generated using adenoviral vector systems. This system used to express NPC1L1 is dervied from Ad 5, a type C adenovirus. This recombinant replication-defective adenoviral vector is made defective through modifications of the E1, E2 and E4 regions. The vector also has additional modifications to the E3 region generally affecting the E3b region genes RIDa and RIDb. NPC1L1 expression was driven using the CMV promoter as an expression cassette substituted in the E3 region of the

adenovirus. Rat and mouse NPC1L1 were amplified using clone specific primers flanked by restriction sites compatible with the adenovirus vector Adenovirus infective particles were produced from 293-D22 cells in titers of 5 X 10^{10} P/mL. Viral lysates were used to infect cells resistant to standard transfection methodologies. In Caco2 cells, which are highly resistant to heterologous protein expression, adenovirus mediated expression of NPC1L1 has been shown by western blot analysis to persist at least 21 days post-infection.

Example 22: NPC1L1 Knock-Out Transgenic Mouse.

[0282] *NPC1L1* knockout mice were constructed via targeted mutagenesis. This methodology utilized a targeting construct designed to delete a specific region of the mouse *NPC1L1* gene. During the targeting process the *E. coli* lacZ reporter gene was inserted under the control of the endogenous *NPC1L1* promoter. The region in *NPC1L1* (SEQ ID NO: 45) being deleted is from nucleotide 790 to nucleotide 998. The targeting vector contains the LacZ-Neo cassette flanked by 1.9 kb 5' arm ending with nucleotide 789 and a 3.2 kb 3' arm starting with nucleotide 999. Genomic DNA from the recombinant embryonic stem cell line was assayed for homologous recombination using PCR. Amplified DNA fragments were visualized by agarose gel electrophoresis. The test PCRs employed a gene specific primer, which lies outside of and adjacent to the targeting vector arm, paired with one of three primers specific to the Lac*Z-Neo* cassette sequence. For 5' PCR reconfirmation, the *NPC1L1* specific oligonucleotide ATGTTAGGTGAGTCTGAACCTACCC (SEQ ID NO: 46) and for 3'PCR reconfirmation the NPC1L1 specific oligonucleotide GGATTGCATTTCCTTCAA GAAAGCC (SEQ ID NO: 47) were used. Genotyping of the F2 mice was performed by multiplex PCR using the NPC1L1 specific forward primer TATGGCTCTGCCC TCTGCAATGCTC (SEQ ID NO: 48) the LacZ-

Neo cassette specific forward primer TCAGCAGCCTCTGTTCCACATACACTTC (SEQ ID NO: 49) in combination with the NPC1L1 gene specific reverse primer GTTCCACAGGGTCTGTGGTGAGTTC (SEQ ID NO: 50) allowed for determination of both the targeted and endogenous alleles. Analysis of the PCR products by agarose gel electrophoresis distinguished the wild-type, heterozygote and homozygote null mouse from each other.

Example 23: Acute Cholesterol Absorption in NPC1L1-Deficient Mice.

[0283] To determine whether NPC1L1 plays a role in cholesterol absorption, *NPC1L1* deficient mice were studied.

[0284] Mice deficient in *NPC1L1* (-/-) were generated by breeding heterozygote mice (+/) to obtain wild-type (+/+) and *NPC1L1* deficient mice (-/-). Non-fasted mice (6.5-9 weeks old, mixed 129 and C57BL/6 background) were weighed and grouped (n=2 -/- and n=4 +/+). All animals were gavaged (Feeding needles, 24G x 1 inch, Popper and Sons, NY) with 0.1 ml corn oil (Sigma; St. Louis, MO) containing 1μCi ^{14}C-cholesterol (New England Nuclear, [$^{4\text{-}14}$C] Cholesterol, NEC-018) and 0.1mg carrier cholesterol mass (Sigma; St. Louis, MO). Two hours later, blood was collected by heart puncture. The liver was removed, weighed, and three samples were placed into 20 ml counting vials. Tissues were digested in 1 ml of 1N NaOH at 60°C overnight. The tissue digests were acidified by addition of 250μl of 4N HCl prior to liquid scintillation counting (LSC). Plasma was isolated by centrifugation at 10,000 rpm for 5 minutes in a microfuge and duplicate 100μl aliquots of plasma were taken for LSC.

[0285] Cholesterol absorption, evaluated by this acute technique and expressed as the total amount of radioactive cholesterol in the plasma and liver, demonstrated that the wild type mice (+/+) absorbed an average of 11,773 dpm and *NPC1L1* deficient mice absorbed 992 dpm of the 14C-cholesterol. These results indicate that the *NPC1L1* deficient mice have a 92% reduction in cholesterol absorption. These data confirm the role of NPC1L1 in intestinal cholesterol absorption. Inhibition of NPC1L1-mediated cholesterol absorption, in a subject, by administering NPC1L1 antagonists, such as ezetimibe, to the subject, are a useful way to reduce serum cholesterol levels and the occurrence of atherosclerosis in the subject.

Example 24: Cholesterol Absorption in *NPC1L1* (*NPC3*) Knockout Mice (Fecal Ratio Method: Cholesterol/Sitostanol).

[0286] In this example, cholesterol absorption and the activity of ezetimibe was determined in the *NPC1L1* knockout mice (-/-), heterozygous mice (+/-), and age matched wild-type mice (+/+).

[0287] Cholesterol absorption in the mice was determined by the dual fecal isotope ratio method as described by Altmann *et al.* (Biochim. Biophys. Acta. 1580(1): 77-93 (2002)). Mice (n= 4-6/group) were fed a standard rodent chow diet and in some groups treated daily with a maximally effective dose of ezetimibe (10 mg/kg). Mice were gavaged with ^{14}C-cholesterol (1μCi, 0.1mg unlabeled cholesterol) and ^{3}H-sitostanol (2μCi) in 0.1ml corn oil. Feces were collected for 2 days and fecal ^{14}C-cholesterol and ^{3}H-sitostanol levels were determined by combustion in a Packard Oxidizer. The fraction of cholesterol absorbed, as evaluated by the fecal dual isotope technique, was similar in wild type (+/+) and heterozygous mice (+/-) fed a chow diet

(heterozygous mice absorbed 46 ±5% and age matched wild type mice absorbed 51 ±3% of the dose of ^{14}C-cholesterol). The *NPC1L1* knockout mice (-/-) absorbed 15.6 ±0.4% of the ^{14}C-cholesterol, which was similar to the wild type mice treated with a maximally effective dose of ezetimibe (16.1 ±0.3%), and reduced by 69% compared to wild type mice ($p<0.001$). In *NPC1L1* knockout treated with ezetimibe at 10 mg/kg/day, cholesterol absorption was similar to that seen in the untreated knockout mice (16.2 ±0.6% compared to 15.6% ±0.4%, respectively). Thus, the majority of cholesterol absorption is dependent on the presence of NPC1L1 and the residual cholesterol absorption in mice lacking NPC1L1 is insensitive to ezetimibe treatment. These results indicate that NPC1L1 is involved in the small intestinal enterocyte uptake and absorption of cholesterol and is in the ezetimibe sensitive pathway.

Example 25: Mouse Screening Assay (Acute Cholesterol Absorption).

[0288] The following screening assay is used to identify the presence of an NPC1L1 antagonist in a sample.

[0289] Mice deficient in *NPC1L1* (-/-) are generated by breeding heterozygote mice (+/) to obtain wild-type (+/+) and *NPC1L1* deficient mice (-/-).

[0290] In a first set of experiments, non-fasted mice (6.5-9 weeks old, mixed 129 and C57BL/6 background) are weighed and grouped (n=1 to 4 -/- and n=1 to 4 +/+). All animals are gavaged (Feeding needles, 24G x 1 inch, Popper and Sons, NY) with 0.1 ml corn oil (Sigma; St. Louis, MO) containing 1μCi ^{14}C-cholesterol (New England Nuclear, [$^{4\text{-}14}$C] Cholesterol, NEC-018) and 0.1mg carrier cholesterol mass (Sigma; St. Louis, MO).

[0291] In another set of experiments, 1 to 4 wild-type *NPC1L1* mice (+/+) are treated identically to the mice in the first set of experiments, above, except that the mice are additionally fed a sample to be tested for the presence of an NPC1L1 antagonist.

[0292] Two hours later, blood is collected from each mouse by heart puncture. The liver is removed, weighed, and three samples are placed into 20 ml counting vials. Tissues are digested in 1 ml of 1N NaOH at 60°C overnight. The tissue digests are acidified by addition of 250µl of 4N HCl prior to liquid scintillation counting (LSC). Plasma is isolated by centrifugation at 10,000 rpm for 5 minutes in a microfuge and duplicate 100µl aliquots of plasma are taken for LSC.

[0293] Cholesterol absorption, evaluated by this acute technique is expressed as the total amount of radioactive cholesterol in the plasma and liver. The sample tested is determined to contain an NPC1L1 antagonist when the level of cholesterol absorption (as measured by the above described methods) in the wild-type *NPC1L1* mouse (+/+) which was fed the sample and in the *NPC1L1* deficient mouse (-/-) are less than the amount of cholesterol absorption in the wild-type *NPC1L1* mouse (+/+) which was not fed the sample.

Example 26: Mouse Screening Assay (Fecal Ratio Method: Cholesterol/Sitostanol).

[0294] The following screening assay is used to identify the presence of an NPC1L1 antagonist in a sample.

[0295] Cholesterol absorption in the mice is determined by the dual fecal isotope ratio method as described by Altmann *et al.* (Biochim. Biophys. Acta. 1580(1): 77-93 (2002)).

[0296] Three groups of mice (n=1-6/group) are assembled. Two separate groups comprise wild-type *NPC1L1* mice (+/+) and one group comprises *NPC1L1* deficient mice (-/-).

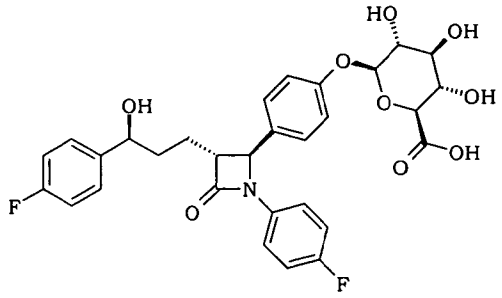
[0297] Each group is fed a standard rodent chow diet and in some groups treated daily. Mice are gavaged with ^{14}C-cholesterol (1μCi, 0.1mg unlabeled cholesterol) and ^{3}H-sitostanol (2μCi) in 0.1ml corn oil. One group of mice, which comprise wild-type NPC1L1 mice (+/+) are further fed a sample to be tested for the presence of an NPC1L1 antagonist. Feces are collected for 2 days and fecal ^{14}C-cholesterol and ^{3}H-sitostanol levels are determined by combustion in a Packard Oxidizer.

[0298] The sample tested is determined to contain an NPC1L1 antagonist when the level of cholesterol and/or sitostanol absorption (as measured by the above described methods) in the wild-type *NPC1L1* mouse (+/+) which was fed the sample and in the *NPC1L1* deficient mouse (-/-) are less than the amount of cholesterol and/or sitostanol absorption in the wild-type *NPC1L1* mouse (+/+) which was not fed the sample.

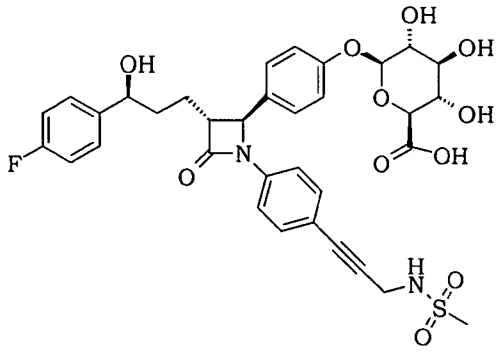
Example 27: Binding Analysis Using Brush Border Membrane Vesicles

[0299] The following screening assay may be used to identify the presence of an NPC1L1 ligand in a sample.

[0300] Materials. The following two compounds were synthesized for the binding assay described herein , ^{3}H-ezetimibe glucuronide 1 (34.5 Ci/mmol) and its ^{35}S-propargyl-sulfonamide derivative 2 (800-1100 Ci/mmol).

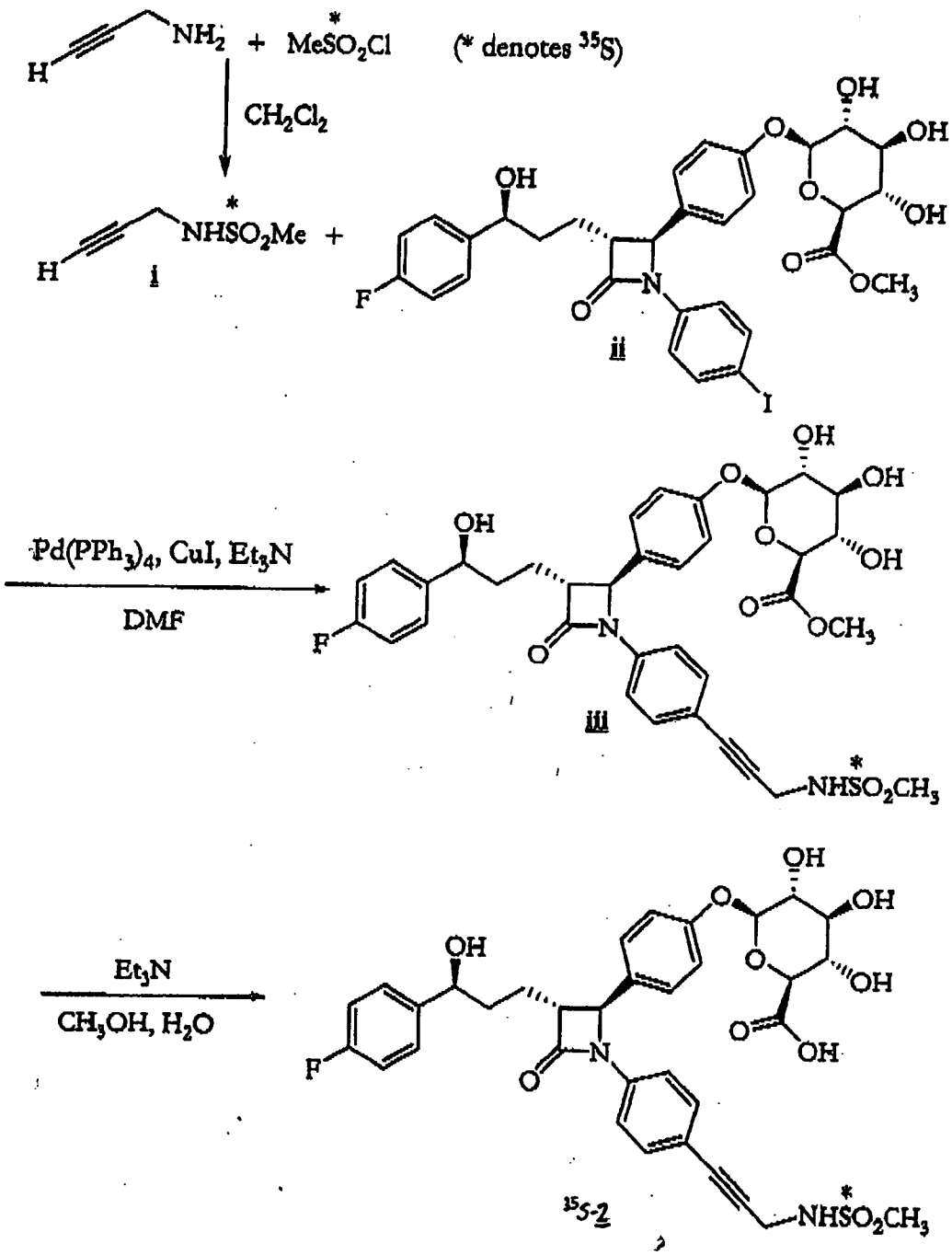


Ezetimibe-glucuronide
1



S-propargyl-sulfonamide
ezetimibe-glucuronide
2

[0301] Synthesis of ezetimibe glucuronide and S-propargyl-sulfonamide ezetimibe-glucuronide. Ezetimibe glucuronide (compound 1) (also referred to as EZE-glucuronide) can be made according to the procedures in U.S. Patent No. 5,756,470. The general scheme below illustrates a method for the synthesis of compound 2 and radiolabelled ^{35}S-2.


NH2
+ MeSO2Cl
(* denotes 35S)
CH2Cl2
NHSO2Me
i
ii
Pd(PPh3)4, CuI, Et3N
DMF
iii
NHSO2CH3
Et3N
CH3OH, H2O
35S-2

[0302] Preparation of compound ^{35}S-**2** (Compound **2** with radiolabelled ^{35}S)

[0303] Step A: Preparation of [^{35}S]N-prop-2-yn-1-ylmethanesulfonamide (**i**). The appropriate volume of [^{35}S]methane sulfonyl chloride (see Dean, D.C.; et al., *J. Med. Chem.* **1996**, *39*, 1767) totaling 3.5 mCi was removed from a stock solution in methylene chloride and placed in a 5mL conical flask. It was then distilled at atmospheric pressure until the volume was approximately 50 μL. To this solution was immediately added 50 μL of propargylamine. After 15 min, the reaction mixture was diluted with 10 mL of ethyl acetate, washed with saturated sodium bicarbonate solution (3 x 2 mL), and dried over sodium sulfate. After filtration the resulting solution had a count of 3.3 mCi and a radiochemical purity of 99.9 % by HPLC (Zorbax XDB C8 column, 4.6 x 150 mm, 5 % acetonitrile:H_2O (0.1 % TFA) to 100 % acetonitrile, 15 min linear gradient, 1 mL/min, t_R = 4.4 min).

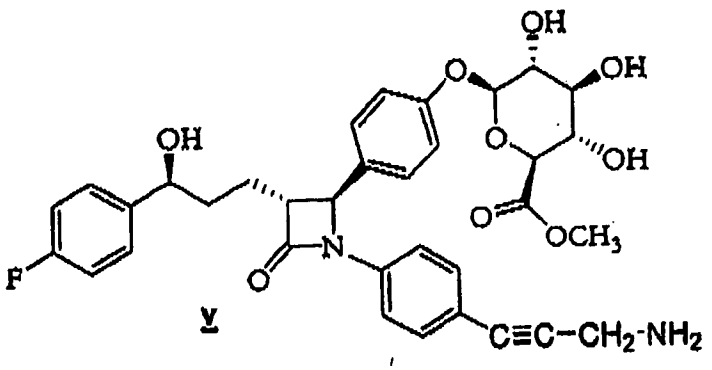
[0304] Step B: Preparation of [^{35}S] 4-[(2*S*,3*R*)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-1-(4-{3-[(methylsulfonyl)amino]prop-1-yn-1-yl}phenyl)-4-oxoazetidin-2-yl]phenyl methyl β-D-glucopyranosiduronate ([^{35}S]) (**iii**). Dissolved 3.0 mCi of [^{35}S]*N*-prop-2-yn-1-ylmethanesulfonamide, 1 mg of compound **ii** (prepared according to Burnett, D.S. et al., Bioorg. Med. Chem. Lett. (2002), vol. 12, p. 311), and 1 μL of triethylamine in 100 μL of dimethylformamide inside a plastic microcentrifuge tube. To this solution was added 10 μL of a stock solution containing 8.1 mg of tetrakis(triphenylphosphine)palladium(0) and 1.4mg of copper iodide in 1 mL of dimethylformamide. Stirred at room temperature for sixty hours at which time HPLC indicated 55% conversion. This reaction mixture, which had a radiochemical purity of 44.4% by HPLC (Zorbax XDB C8 column, 4.6 x 150 mm, 5 % acetonitrile:H_2O (0.1 %

TFA) to 100 % acetonitrile, 15 min linear gradient, 1 mL/min, t_R = 9.3 min) was taken on directly to the next step.

[0305] Step C:Preparation of [^{35}S] 4-[(2S,3R)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-1-(4-{3-[(methylsulfonyl)amino]prop-1-yn-1-yl}phenyl)-4-oxoazetidin-2-yl]phenyl β-D-glucopyranosiduronic acid ^{35}S-**2**. The crude reaction mixture containing compound **iii** was treated with 25 μL of methanol, 90 μL of water, and 30 μL of triethylamine and stirred at room temperature for one hour at which time it was concentrated to near dryness under a slow stream of nitrogen. The residue was dissolved in 1:1 acetonitrile:H_2O and subjected to semi-preparative chromatography (Zorbax XDB C8 250 x 9.4 mm column, 70:30 acetonitrile:H_2O (0.1 % TFA) 4 mL/min, 1 x 0.2 mL injections). 540 μCi of product was obtained which had a radiochemical purity of 99.9% by HPLC (Zorbax XDB C8 column, 4.6 x 150 mm, 70:30 acetonitrile:H_2O (0.1 % TFA), 1 mL/min, t_R = 10.4 min) and coeluted with an authentic sample of compound **2**. LC/MS m/z = 508 (product – glucuronide – H_2O), SA = 769 Ci/mmol.

Alternate Preparation of ^{35}S-2.

[0306] Step A: Preparation of **iii.** The appropriate volume of [^{35}S]methane sulfonyl chloride (see Dean, D.C.; et al., *J. Med. Chem.* **1996**, *39*, 1767) totaling 1.3 mCi was removed from a stock solution in methylene chloride and placed in a 5mL conical flask. It was then distilled at atmospheric pressure until the volume was approximately 50 μL. To this solution was immediately added a solution of 1 mg of **v** in 5 μL of pyridine (freshly distilled over calcium hydride).



v

[0307] The solution was stirred at room temperature for five minutes at which time it was concentrated to near dryness under a slow stream of nitrogen. This reaction mixture, which had a radiochemical purity of 80.1% by HPLC (Zorbax XDB C8 column, 4.6 x 150 mm, 5% acetonitrile:H_2O (0.1 % TFA) to 100 % acetonitrile, 15 min linear gradient, 1 mL/min, t_R = 9.3 min) was taken on directly to the next step.

[0308] Step B:Preparation of ^{35}S-**2**. The crude reaction mixture containing **iii** was treated with 25 μL of methanol, 90 μL of water, and 30 μL of triethylamine and stirred at room temperature for one hour at which time it was concentrated to near dryness under a slow stream of nitrogen. The residue was dissolved in 1:1 acetonitrile:H_2O and subjected to semi-preparative chromatography (Zorbax XDB C8 250 x 9.4 mm column, 70:30 acetonitrile:H_2O (0.1 % TFA) 4 mL/min, 1 x 0.2 mL injections). 350 μCi of product was obtained which had a radiochemical purity of 98.4 % by HPLC (Zorbax XDB C8 column, 4.6 x 150 mm, 70:30 acetonitrile:H_2O (0.1 % TFA), 1 mL/min, t_R = 10.4 min) and coeluted with an authentic sample of **2**. LC/MS m/z = 508 (product – glucuronide – H_2O), SA = 911 Ci/mmol.

[0309] Following the same general procedure for synthesis of ^{35}S-**2**, except omitting the radiolabelling, compounds **2** and **iv** can be prepared.

[0310] Preparation of brush border membrane vesicles (BBMV). Membranes were prepared from Rhesus macaque (Macaca mulatta), rat (male Sprague-Dawley), and mouse (male C57BL/6J) intestines, using Mg^{++} precipitation method described in the following references and with modifications described below (Hauser, H., Howell, K., Dawson, R.M.C., Bowyer, D. E. Biochim. Biophys. Acta 602, 567-577 (1980); Kramer, W., Girbig, F., Gutjahr, U., Kowalewski, S., Jouvenal, K., Muller, G., Tripier, D., Wess, G. J. Biol. Chem. 268, 18035-18046 (1993); Rigtrup, K.M., Ong, D.E. Biochemistry 31, 2920-2926 (1992)).

[0311] The intestines from freshly sacrificed animals were cut into segments, perfused with ice-cold saline buffer (Buffer A: 26 mM $NaHCO_3$, 0.96 mM NaH_2PO_4, 5 mM HEPES, 5.5 mM glucose, 117 mM NaCl, 5.4 mM KCl, pH = 7.4), placed on cold glass plates, opened longitudinally, and the mucosa scraped with glass microscope slips. This mucosa could be used fresh or frozen with identical results. To prepare the membranes, the mucosal scrapings were resuspended in 20 volumes of cold buffer consisting of 300 mM D-mannitol, 5 mM EGTA, 12 mM Tris, pH 7.4 with HCl, and containing 0.1 mM PMSF and a 1% dilution of a protease inhibitor cocktail (set 1, Calbiochem). They were homogenized using a Polytron at medium speed on ice until inspection with a microscope indicated complete cell lysis. Then, solid $MgCl_2$ was added slowly with stirring to a final concentration of 10 mM, and the solution was kept stirring on ice for 15 min. Cellular debris was removed by centrifugation for 15 min at 3,000g, and the membranes were recovered by centrifugation for 60 min at 48,000g. The membranes were further rinsed by re-suspension in a buffer containing 50 mM D-mannitol, 5 mM EGTA, and 2 mM Tris at pH 7.40, and centrifugation for 60 min at 48,000g. The final pellet was resuspended in 120 mM NaCl and 20 mM Tris at pH 7.40 to a concentration of ~10-20 mg

protein/ml, aliquoted, frozen in liquid nitrogen, and stored at –80C. The activity was stable indefinitely and could be freeze-thawed with minimal loss.

[0312] Membrane protein was measured by the Bradford assay (Bradford, M.M. Anal. Biochem. 72, 248-254 (1976)) using bovine serum albumin as standard. The enrichment in brush border membranes was assessed using gamma-glutamyltransferase as a marker enzyme (Kramer, W., Girbig, F., Gutjahr, U., Kowalewski, S., Jouvenal, K., Muller, G., Tripier, D., Wess, G. J. Biol. Chem. 268, 18035-18046 (1993)), which indicated a 6-fold enrichment over the initial homogenate.

[0313] Binding assay. Assays were conducted in 12 x 75 mm glass test tubes and total volume 100 ul. In general, frozen membranes were diluted in buffer A to a final concentration of 0.02 to 5 mg/ml. Radiolabeled ligands were typically 25-50 nM for ^{3}H-ezetimibe (EZE)glucuronide **1** (200,000-400,000 dpm), and 3-5 nM for its ^{35}S analog **2**, (300,000-1,000,000 dpm) in the assay, and they were delivered as DMSO or CH_3CN solutions. Competing ligands were likewise added as DMSO solutions to give a total 2-10 % organic solvent content. Nonspecific binding was defined by competition with 100 uM ezetimibe glucuronide. At least 2 components of buffer A, the bicarbonate and phosphate salts, were later found to be inconsequential and were routinely omitted. To ensure equilibrium was established, the reactions with compound **1** were incubated at least 3 hours for rhesus membranes and at least one hour for rat membranes at room temperature, and with compound **2** as long as 2 hours at 37°C with both rhesus and rat membranes.

[0314] Bound ligand was quantified by single-tube vacuum filtration using GF/C glass fiber filters. Glass fiber filters (GF/C) were obtained from Whatman. The filters were pretreated by soaking with 0.5% polyethylenimine to reduce nonspecific binding. Filtration was accomplished by adding 2.5 ml of ice cold buffer (120 mM NaCl, 0.1% sodium cholate, and 20 mM MES at pH 6.70) to the assay tube, pouring the mixture through the filter, and then rinsing the tube and filter twice more with another 2 x 2.5 ml buffer. The filters were counted in 7 ml vials using Packard DM liquid scintillation fluid. Where triplicate assays were performed, the standard error was typically <4%. As an example, assay of rat brush border membranes at 2 mg/ml in the presence of 400,000 dpm (50 nM) ^{3}H-ezetimibe glucuronide gave 15,000 dpm specific and 3,000 dpm nonspecific binding. The filters contributed most of the nonspecific binding (2,000 dpm).

[0315] Alternatively, vacuum filtration of compound **2** on a 96-well plate (Whatman GF/C) can also be used to achieve adequate precision.

[0316] Data analysis. Data from saturation experiments were subjected to a Scatchard analysis, and linear regression was performed to yield the equilibrium dissociation constant (K_d) and maximum receptor concentration (B_{max}). Correlation coefficients for these determinations were typically greater than 0.96. Data from competition experiments were analyzed and IC_{50} values determined from Hill plots of the binding data. The kinetic data for ligand association and dissociation were subjected to the analysis of Weiland and Molinoff (Weiland, G., Molinoff, .B. Life Sci. 29, 313-330 (1981)). The dissociation rate constant for (k_{-1}) was determined directly for a first order plot of ligand dissociation versus time. The rate of ligand association (k_1) was determined from the equation $k_1 = k_{obs}([LR_e]/([L]\ [LR]_{max}))$ where [L] is the concentration of

ligand, $[LR_e]$ is the concentration of the complex at equilibrium, $[LR]_{max}$ is the maximum number of receptors present, and k_{obs} is the slope of the pseudo-first order plot L_n ($[LE]_e/([LR]_e - [LR]_t)$) versus time.

[0317] Binding analysis. Ezetimibe is rapidly converted to its glucuronide *in vivo*, and this metabolite is thought to be largely if not exclusively responsible for inhibition of cholesterol uptake. Accordingly, both ^{3}H-ezetimibe and its corresponding glucuronide derivative (**1**) were prepared and tested for binding to intestinal brush border membrane preparations, using a single-tube vacuum filtration technique. As a result of the hydrophobic nature of ^{3}H-ezetimibe, high nonspecific binding was observed, precluding its use as a radioligand for the binding assay. However, due to the improved physical properties of the glucuronide derivative (**1**), specific binding was observed with this radioligand and it was used to assess binding in rhesus, rat, and mouse intestinal brush border membranes.

[0318] Rhesus, rat, and mouse intestinal scrapings were homogenized and the brush border membranes isolated. Specific binding was observed exclusively in the membrane fraction. Plots of total, nonspecific, and specific binding to rhesus (Figure 1) and rat (Figure 2) brush border membranes. Aliquots of rhesus BBMV (83 μg/assay) or rat BBMV (250μg/assay) were incubated with increasing concentrations of ^{3}H-EZE-glucuronide. Total binding and nonspecific binding determined in the presence of 10-100 uM EZE-glucuronide are shown. Specific binding was calculated from the difference between total and nonspecific binding. Data were fit by nonlinear regression as described above, and the linear Scatchard plot is shown. In rhesus membranes, the data correspond to a single binding site with K_d = 41 nM and a concentration of

5.5 pmol/mg membrane protein. The affinity is ~10-fold lower in rat membranes (K_d = 540 nM). ^{3}H-EZE-glucuronide was not useful as a ligand for a binding assay for the mouse target due to the compounds low affinity in mouse membrane. These potencies correlate roughly with the sensitivity of these species to ezetimibe inhibition of cholesterol uptake.

[0319] Rate constants for binding and dissociation. Ezetimibe-glucuronide is slow to bind, and forms a relatively long-lived complex with its receptor. Indeed, this was key to detecting the interaction in a traditional filter-binding assay, as ligand/receptor interactions with K_d values greater than 100 nM often go unrecognized because of the typical fast off-rates of the ligands. Rate constants for association (k_{on}) and dissociation (k_{off}) of compound **1** were determined for rat and rhesus membranes, and used as an alternative method to calculate the dissociation constant (K_d) according to the relationship $K_d = k_{off}/k_{on}$. 300μg/assay of rat brush border membrane vesicles were incubated with 25nM ^{3}H-EZE-glucuronide at room temperature for up to three hours for the association kinetic studies. 83 μg/assay of rhesus brush border membrane vesicles were incubated with 25nM ^{3}H-EZE-glucuronide at room temperature for up to five hours for the association kinetic studies. Nonspecific binding measured in the presence of 100μM EZE-glucuronide was substracted from the total binding to calculate the specific binding shown in Figures 3A and 4A. For the dissociation kinetic study, rat brush border membrane vesicles were incubated with 25nM ^{3}H-EZE-glucuronide for 2 hours at room temperature and ligand dissociation was initiated by the addition of 100μM EZE-glucuronide. Rhesus brush border membrane vesicles were incubated with 42nM ^{3}H-EZE-glucuronide for 4 hours at room temperature and ligand dissociation was initiated by the addition of 100μM EZE-glucuronide.

For both rat and rhesus dissociation studies, samples were collected at various times and radiolabel was detected. Dissociation curves are shown in Figures 3B (rat) and 4B (rhesus).

[0320] For rat membranes, the rate constant for association is k_{on} = 5,540 M^{-1} s^{-1} (compared to 10^8 to10^9 M^{-1} s^{-1} for diffusion controlled encounter), and the rate constant for dissociation is k_{off} = 2.4 x 10^{-3} s^{-1}, corresponding to a half-life of 4.7 min. The data are shown in Figure 3, where the solid lines are theoretical for these rate constants. The K_d value predicted from these rate constants ($K_d = k_{off}/k_{on}$ = 440 nM) agrees well with that measured at equilibrium (K_d = 540 nM).

[0321] For rhesus membranes, where ^{3}H-ezetimibe glucuronide is at least 10-fold more potent (as described above), the association rate remains the same but the half-life for dissociation of the complex increases to ~90 min. These data are shown in Figure 4, where the theoretical lines correspond to k_{on} = 3,900 M^{-1} s^{-1} and k_{off} = 1.23 x 10^{-4} s^{-1}, and predict K_d = 32 nM compared to that measured at equilibrium (K_d = 41 nM).

Example 28: Binding Analysis of a Potent NPC1L1 ligand

[0322] A ^{35}S-labeled propargyl-sulfonamide analogue of ezetimibe glucuronide (^{35}S-**2**) was identified as a potential NPC1L1 antagonist. Compound **2** was prepared as described in Example 27 and found to have markedly improved affinity for some species of brush border membranes vesicles. For rhesus brush border membranes vesicles, 56 μg protein/assay were incubated with 25 nM ^{3}H-EZE-glucuronide in the presence of increasing concentrations of EZE-glucuronide and **2**. For rat brush border membranes vesicles, 150 μg protein/assay were incubated with 50 nM ^{3}H-EZE-glucuronide in the presence of increasing concentrations of EZE-glucuronide and **2**. For mouse brush border membranes vesicles, 20 μg protein/assay were

incubated with 3 nM ^{35}S-2 in the presence of increasing concentrations of EZE-glucuronide and 2.

[0323] 2 is more potent against enterocyte brush border membrane preparations from rats (35-fold), but is equipotent with ezetimibe glucuronide for rhesus membrane preparations (Figure 5, Table 8). It also has enhanced affinity for mouse membranes (Figure 6, Table 8).

Table 8. Summary of dissociation constants (Kd) for binding of ezetimibe glucuronide 1 and its propargyl-sulfonamide derivative 2 to rhesus, rat, and mouse intestinal brush border membranes.

Compound	Rhesus	Rat	Mouse
1	39	530	2,300
2	38	15	144

Kd values are nM.

Example 29: Distribution of ^{3}H-ezetimibe glucuronide (1) binding to intestinal tissues.

[0324] Previous studies have established that cholesterol absorption occurs primarily in the jejunum, and is substantially lower in the ileum and duodenum. To determine if the binding activity is similarly distributed, the binding assay using ^{3}H-ezetimibe glucuronide (^{3}H-1) as a radioligand was used to determine the distribution of binding sites in sections from rhesus and rat intestines.

[0325] For the rhesus studies, 10 cm corresponding to the ileum of a rhesus small intestine was separated and the remaining intestine was divided into three segments, (proximal, middle and distal) of equal length (70 cm each). For the rat studies, 10 cm corresponding to the ileum of a

rhesus small intestine was separated and the remaining intestine was divided into three segments, (proximal, middle and distal) of equal length (36 cm each). Brush border membrane vesicles were prepared as described in Example 27. Aliquots of vesicles (100-200μg) protein/assay were incubated with 50nM ^{3}H-EZE-glucuronide in the absence and presence of 100μM EZE-glucuronide.

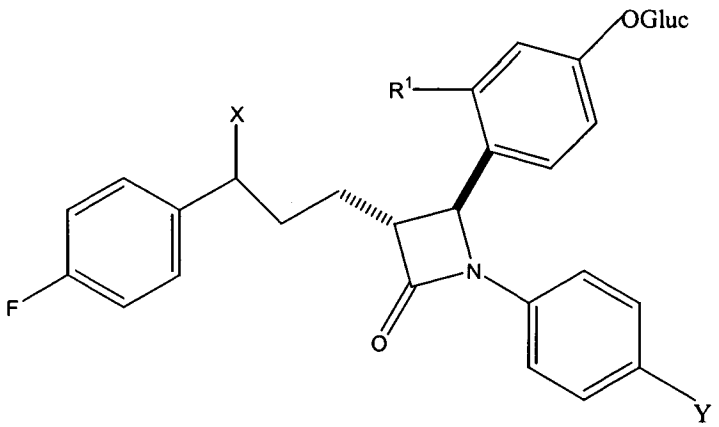
[0326] As shown in Figure 7, specific binding for ^{3}H-ezetimibe glucuronide peaks in the jejunum in both species, consistent with the previously observed pattern of cholesterol absorption.

Example 30: Correlation of *in vitro* and *in vivo* binding activity of NPC1L1

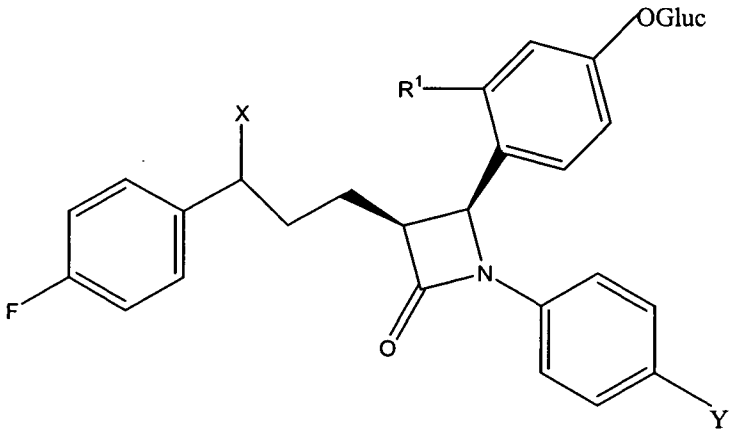
[0327] To determine if *in vitro* binding activity is predictive of *in vivo* efficacy, the enantiomer of ezetimibe glucuronide and several close structural analogues of ezetimibe glucuronide that were tested in the rat membrane binding assay were tested in an acute rat cholesterol absorption study as described in Examples 23-26. The selected analogs had a range of *in vitro* potencies, and were anticipated to have similar physical properties to ezetimibe glucuronide (Table 9). The enantiomer, which has a Kd > 100,000 nM for the rat target, was inactive in the *in vivo* assay. For the other analogs, the same rank order of potency is observed in the *in vitro* and *in vivo* assays, further evidence that the observed binding is due to the target of ezetimibe.

Table 9. IC_{50} values of EZE-gluc and analogs to inhibit binding of 3H-EZE-gluc to rat brush border membrane vesicles.

Compound Name	R^1	X	Y	RAT IC_{50} (nM)
3	H	H2	F	2,300
EZE-gluc 1	H	(S)-OH	F	530
EZE-gluc enantiomer 4	H	(R)-OH	F	>100000
5	H	(R)-OH	F	3,900
6	H	=O	F	70,000
7	OH	(S)-OH	F	252



Backbone structure for compounds 1, 3, 5, 6 and 7.



Backbone structure for compound 4.

Example 31: Binding affinities of ezetimibe glucuronide and its analogs to recombinant NPC1L1

[0328] NPC1L1 was identified as a candidate target of ezetimibe from a search of genetic databases for cholesterol binding motifs. Subsequently, *NPC1L1* deficient mice were found to have 80% reduction of cholesterol absorption, and did not respond to ezetimibe treatment, strongly suggesting that this protein is required for ezetimibe efficacy. To determine if NPC1L1 is the direct target of ezetimibe, binding affinities were compared for ezetimibe glucuronide and several analogs to NPC1L1 transfected cells and rat brush border membrane vesicles.

[0329] Rat NPC1L1 transfected CHO cells (~500,000 cells/assay) were incubated with 5 nM ^{35}S-**2** (~1 million dpm/assay) for 2 hours at 37 °C in the absence or presence of increasing concentrations of EZE-glucuronide (compound **1**), compounds **2**, **3**, **5**, **6**, or **8**.

[0330] Human NPC1L1 transfected CHO cells (~600,000 cells/assay) were incubated with 5 nM ^{35}S-**2** (~1 million dpm/assay) in buffer A for 2 hours at 37 °C in the absence or presence of increasing concentrations of EZE-glucuronide (compound **1**), compounds **2**, **3**, **5**, **6**, or **8**.

[0331] As shown in Figure 8 and Table 10, the affinities for the recombinant and native proteins are virtually identical, providing compelling evidence that NPC1L1 is the direct target of ezetimibe in rodent tissues, and that other proteins are not required for binding.

[0332] Affinities of ezetimibe glucuronide and analogues thereof were also determined for human recombinant NPC1L1. The results, shown in Figure 9, indicate that ezetimibe

glucuronide (1) has an affinity for the human protein of 907 nM. The propargyl-sulfonamide analogue (2) is approximately 50-fold more potent, with a K_d = 21 nM, suggesting that this compound has the potential for enhanced potency of cholesterol absorption inhibition in man.

Table 10: Comparison of dissociation constants (Kd) for binding to native rat intestinal brush border membranes and membranes from rat NPC1L1 transfected cells.

Analog	Recombinant rat NPC1L1 Kd, nM	Rat BBMV Kd, nM
EZE-glucuronide (1)	790	600
2	12	15
3	2400	2300
6	84500	70000
5	5800	3900
8	556	818

Example 32: Binding of ^{35}S-2 to membranes from wild type and NPC1L1 deficient mice.

[0333] Final confirmation that NPC1L1 is the target of ezetimibe was provided by binding studies with ^{35}S-2 in intestinal brush border membranes from NPC1L1 deficient and control mice.

[0334] Brush border membranes vesicles were prepared from intestinal tissues of wild type and *NPC1L1* knockout (-/-) mice. 15, 30 and 60μg protein/ assay of brush border membranes vesicles were incubated with 4nM ^{35}S-2 in buffer A for 3 hours at 37°C in the presence and absence of 100μM EZE-glucuronide.

[0335] The results, shown in Figure 10, indicate that no detectable binding is observed in membranes from NPC1L1 deficient mice, whereas age matched wild type control membranes have detectable binding. The binding affinity observed in this experiment in control mouse membranes (K_d=156 nM) was virtually identical to that observed in previous studies (Figure 11).

****

[0336] The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

[0337] Patents, patent applications, publications, product descriptions, Genbank Accession Numbers and protocols are cited throughout this application, the disclosures of which are incorporated herein by reference in their entireties for all purposes.

I Claim:

1. A method for identifying a ligand of NPC1L1 comprising:

contacting NPC1L1 with a detectably labeled substituted 2-azetidinone and a candidate compound; and

determining whether said candidate compound binds to NPC1L1;

wherein binding of said compound to NPC1L1 modulates binding of said detectably labeled substituted 2-azetidinone to NPC1L1 and

wherein said modulation indicates that the candidate compound is a ligand that binds to NPC1L1.

2. The method of claim 1, wherein NPC1L1 has an amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 12 and a fragment thereof.

3. The method of claim 1, wherein binding of said substituted 2-azetidinone to NPC1L1 is disrupted.

4. The method of claim 1, wherein NPC1L1 is membrane-bound.

5. The method of claim 4, wherein the membrane is isolated from mammalian intestines.

6. The method of claim 5, wherein the membrane is a brush border membrane.

7. The method of claim 6, wherein the brush border membrane is from the group consisting of rhesus, rat, murine, and human intestinal tissue.

8. The method of claim 4, wherein the membrane is vesicularized.

9. The method of claim 4, wherein the membrane is isolated from cultured cells.

10. The method of claim 9, wherein the membrane is isolated from recombinant NPC1L1-expressing cells.

11. The method of claim 1, wherein NPC1L1 is solubilized.

12. The method of claim 11, wherein NPC1L1 is isolated from membranes derived from mammalian intestines.

13. The method of claim 12, wherein the membranes are brush border membranes.

14. The method of claim 13, wherein the brush border membranes are from the group consisting of rhesus, rat, murine, and human intestinal tissue.

15. The method of claim 1, wherein the detectably labeled substituted 2-azetidinone is selected from the group consisting of ^{3}H, ^{35}S, ^{125}I, and a fluorescently labeled substituted 2-azetidinone.

16. The method of claim 1, further comprising incubating NPC1L1 with said detectably labeled substituted 2-azetidinone and said candidate compound.

17. The method of claim 15, wherein the binding of said detectably labeled substituted 2-azetidinone to NPC1L1 is detected by liquid scintillation counting.

18. The method of claim 17, wherein NPC1L1 bound to detectably labeled substituted 2-azetidinone is collected onto filters by vacuum filtration.

19. The method of claim 17, wherein the binding of said detectably labeled substituted 2-azetidinone to NPC1L1 is detected using a scintillation proximity assay.

20. The method of claim 1, wherein said detectably labeled substituted 2-azetidinone is a substituted 2-azetidinone-glucuronide.

21. The method of claim 20, wherein the substituted 2-azetidinone-glucuronide is selected from the group consisting of ezetimibe-glucuronide and compound **2**.

22. The method of claim 21, wherein said substituted 2-azetidinone-glucuronide is ezetimibe-glucuronide.

23. The method of claim 21, wherein said substituted 2-azetidinone-glucuronide is compound **2**.

24. A method for identifying a compound that inhibits intestinal sterol or 5α-stanol absorption, wherein said absorption is mediated by NPC1L1, comprising

contacting NPC1L1 with a detectably labeled ligand and a candidate compound; and

determining whether said candidate compound binds to NPC1L1;

wherein binding of said candidate compound to NPC1L1 modulates binding of said ligand to NPC1L1,

wherein said modulation indicates that the candidate compound is an intestinal sterol or 5α-stanol absorption inhibitor.

25. The method of claim 24, wherein the sterol is selected from the group consisting of cholesterol, sitosterol, campesterol, stigmasterol, avenosterol and a cholesterol oxidation product, and the 5α-stanol is selected from the group consisting of cholestanol, 5α-campesterol, and 5α-sitostanol.

26. A method for identifying a ligand of NPC1L1 comprising:

contacting NPC1L1 with a detectably labeled substituted 2-azetidinone; and

measuring binding of NPC1L1 with the detectably labeled substituted 2-azetidinone in the presence and absence of a candidate compound;

wherein decreased binding of the detectably labeled substituted 2-azetidinone to the NPC1L1 in the presence of the candidate compound indicates that said candidate compound is a ligand of NPC1L1.

27. The method of claim 26, wherein NPC1L1 has an amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 12 and a fragment thereof.

28. The method of claim 27, wherein NPC1L1 is membrane-bound.

29. The method of claim 28, wherein the membrane is isolated from mammalian intestines.

30. The method of claim 29, wherein the membrane is a brush border membranes.

31. The method of claim 30, wherein the brush border membrane is from the group consisting of rhesus, rat, murine, and human intestinal tissue.

32. The method of claim 28, wherein the membrane is vesicularized.

33. The method of claim 28, wherein the membrane is isolated from cultured cells.

34. The method of claim 33, wherein the membrane is isolated from recombinant NPC1L1-expressing cells.

35. The method of claim 27, wherein NPC1L1 is solubilized.

36. The method of claim 35, wherein NPC1L1 is isolated from membranes derived from mammalian intestines.

37. The method of claim 36, wherein the membranes are brush border membranes.

38. The method of claim 37, wherein the brush border membranes are derived from the group consisting of rhesus, rat, murine, and human intestinal tissue.

39. The method of claim 26, wherein the detectably labeled substituted 2-azetidinone is selected from the group consisting of ^{3}H, ^{35}S, ^{125}I, and a fluorescently labeled substituted 2-azetidinone.

40. The method of claim 26, further comprising incubating NPC1L1 with said detectably labeled substituted 2-azetidinone and said candidate compound.

41. The method of claim 39, wherein the binding of said detectably labeled substituted 2-azetidinone to NPC1L1 is detected by liquid scintillation counting.

42. The method of claim 41, wherein NPC1L1 bound to detectably labeled substituted 2-azetidinone is collected onto filters by vacuum filtration.

43. The method of claim 41, wherein the binding of said detectably labeled substituted 2-azetidinone-glucuronide to NPC1L1 is detected using a scintillation proximity assay.

44. The method of claim 26, wherein said detectably labeled substituted 2-azetidinone is a substituted 2-azetidinone-glucuronide.

45. The method of claim 44, wherein the substituted 2-azetidinone-glucuronide is selected from the group consisting of ezetimibe-glucuronide and compound **2**.

46. The method of claim 45, wherein said detectably labeled substituted 2-azetidinone-glucuronide is ezetimibe-glucuronide.

47. The method of claim 45, wherein said detectably labeled substituted 2-azetidinone-glucuronide is compound 2.

48. A method for identifying a compound that inhibits intestinal sterol or 5α-stanol absorption wherein said absorption is mediated by NPC1L1 comprising

contacting NPC1L1 with a detectably labeled ligand and the candidate compound;

measuring binding of NPC1L1 with the detectably labeled ligand in the presence and absence of a candidate compound;

wherein binding of said candidate compound to NPC1L1 modulates binding of said ligand to NPC1L1,

wherein said binding indicates that the candidate compound is an intestinal steroid or 5α-steroid absorption inhibitor.

49. The method of claim 48, wherein the sterol is selected from the group consisting of cholesterol, sitosterol, campesterol, stigmasterol, avenosterol and a cholesterol oxidation product, and the 5α-stanol is selected from the group consisting of cholestanol, 5α-campesterol, and 5α-sitostanol.

ABSTRACT OF THE DISCLOSURE

The present invention provides human, rat and mouse NPC1L1 polypeptides and polynucleotides encoding the polypeptides. Methods for detecting ligands which bind to NPC1L1 and block intestinal cholesterol absorption are provided. Also included is a method of identifying ligands which bind to NPC1L1 using membranes derived from brush border membrane preparations. Compounds that bind to NPC1L1 can be used for inhibiting intestinal cholesterol absorption in a subject.

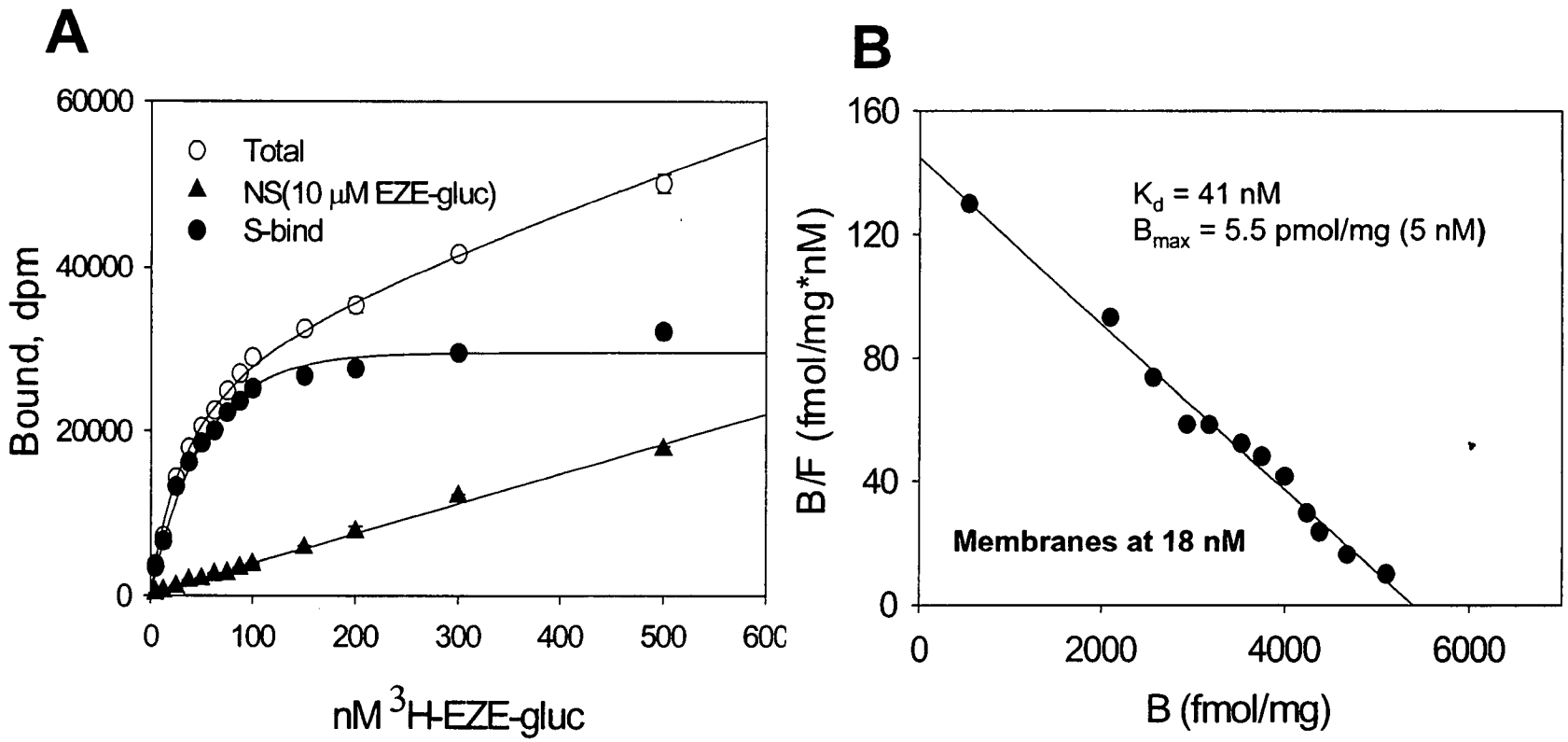


Figure 1. Equilibrium binding of EZE-glucuronide to rhesus BBMVs

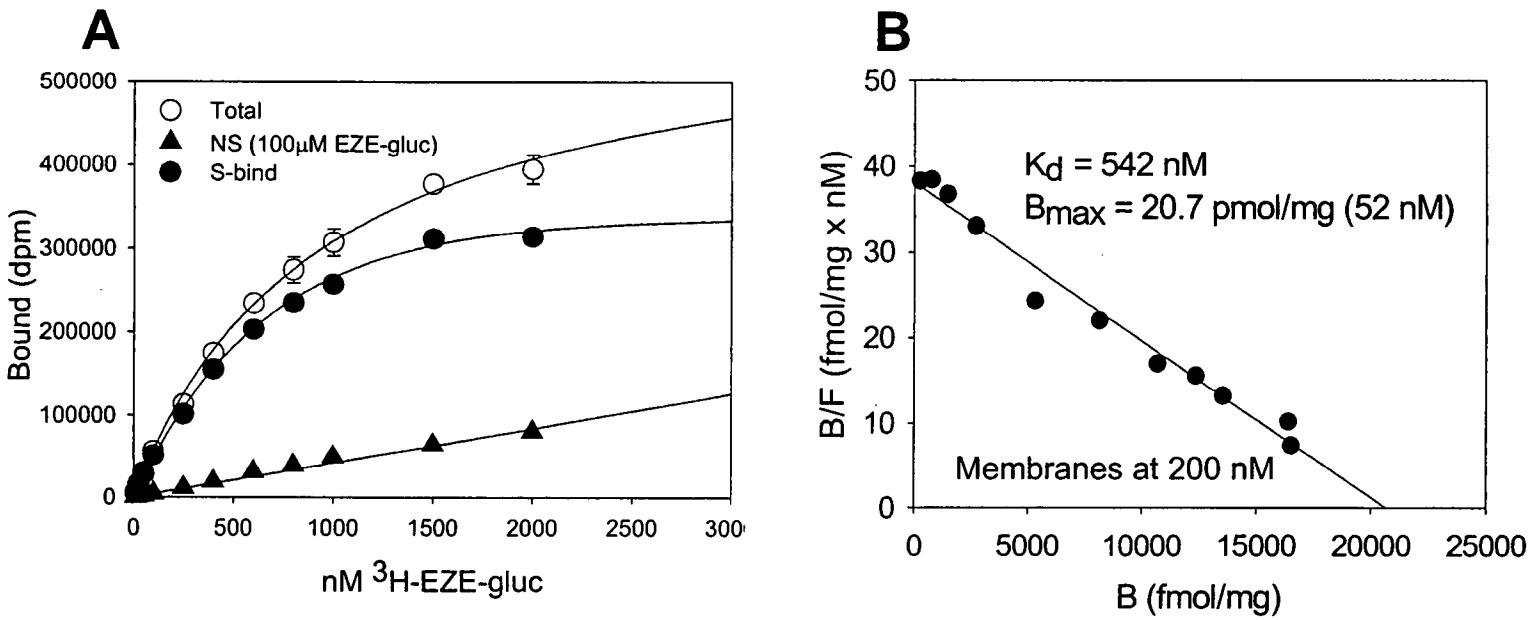
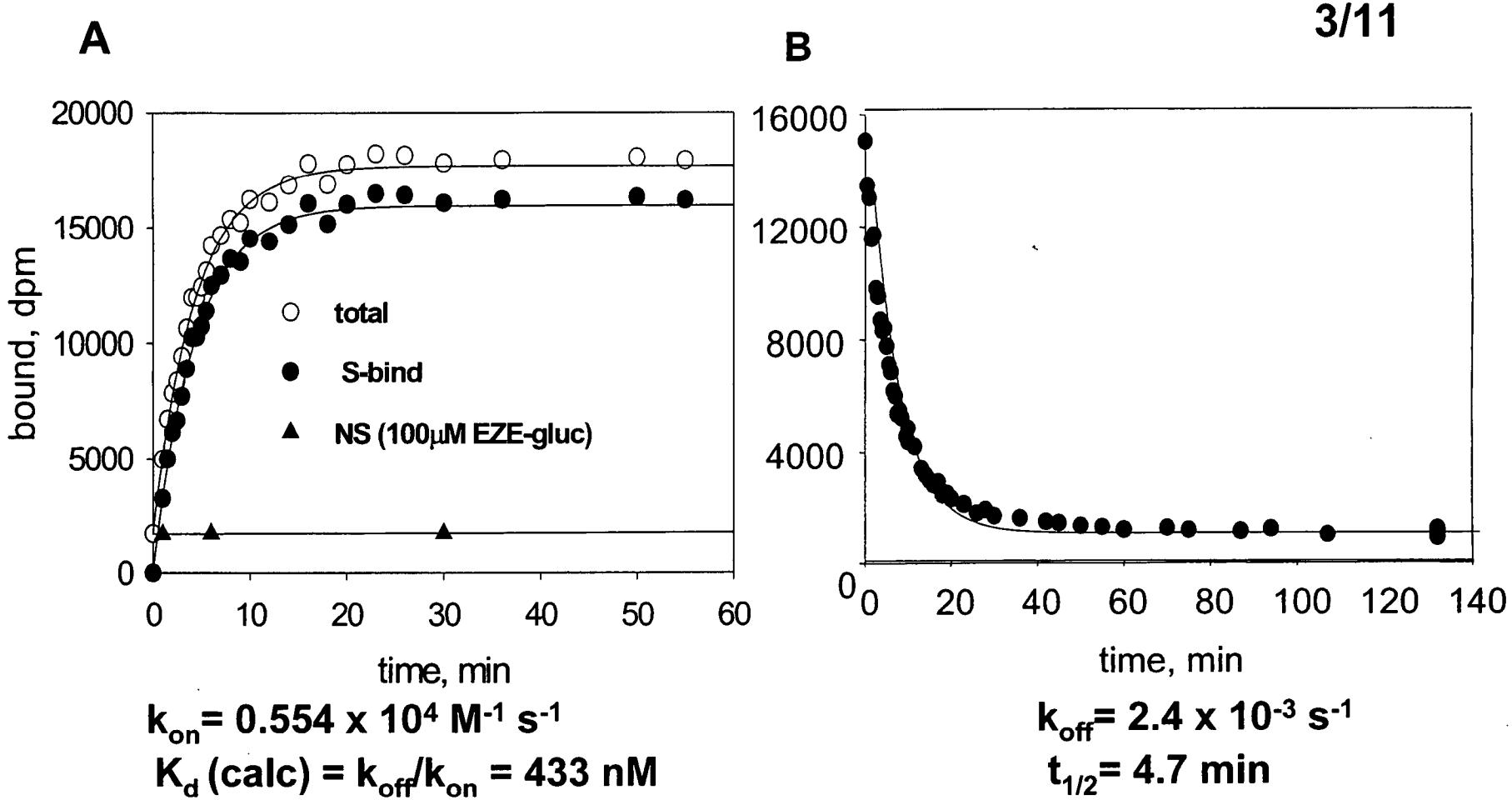


Figure 2. Equilibrium binding of EZE-glucuronide to rat BBMV.



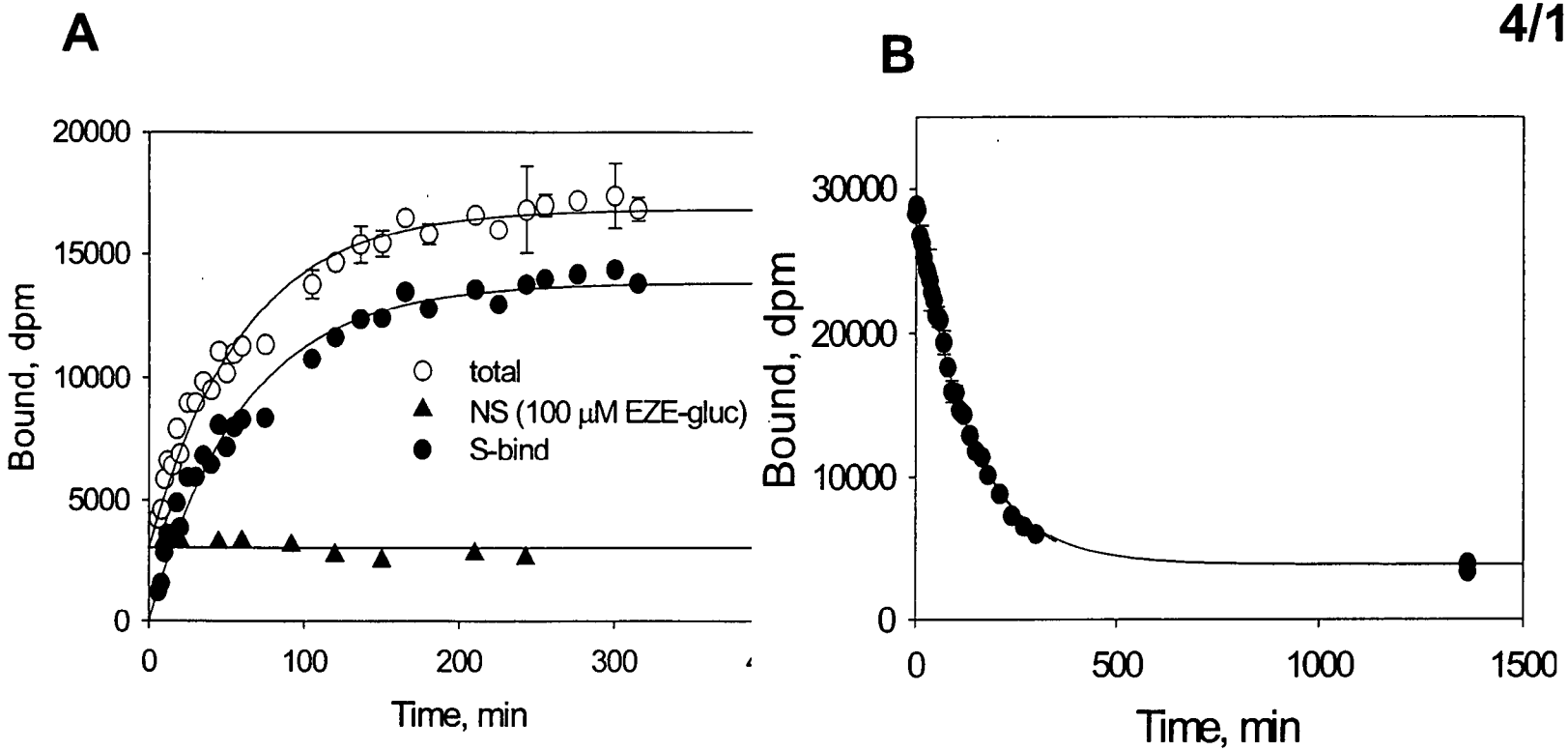
k_{on}= 0.554 x 10^4 M^{-1} s^{-1}

K_d (calc) = k_{off}/k_{on} = 433 nM

k_{off}= 2.4 x 10^{-3} s^{-1}

$t_{1/2}$= 4.7 min

Figure 3. Association and dissociation kinetics of ^{3}H-EZE-glucuronide in rat BBMV.



kobs = 0.01673 min-1 = 2.79 x 10-4 s-1

k_{off}= 1.23 x 10^{-4} s^{-1}

k_{on}= 3.9 x 10^{3} M^{-1} s^{-1}

$t_{1/2}$= 94 min

K_d (calc) = k_{off}/k_{on} = 32 nM

Figure 4. Association and Dissociation kinetics of ^{3}H-EZE-glucuronide in rhesus BBMV.

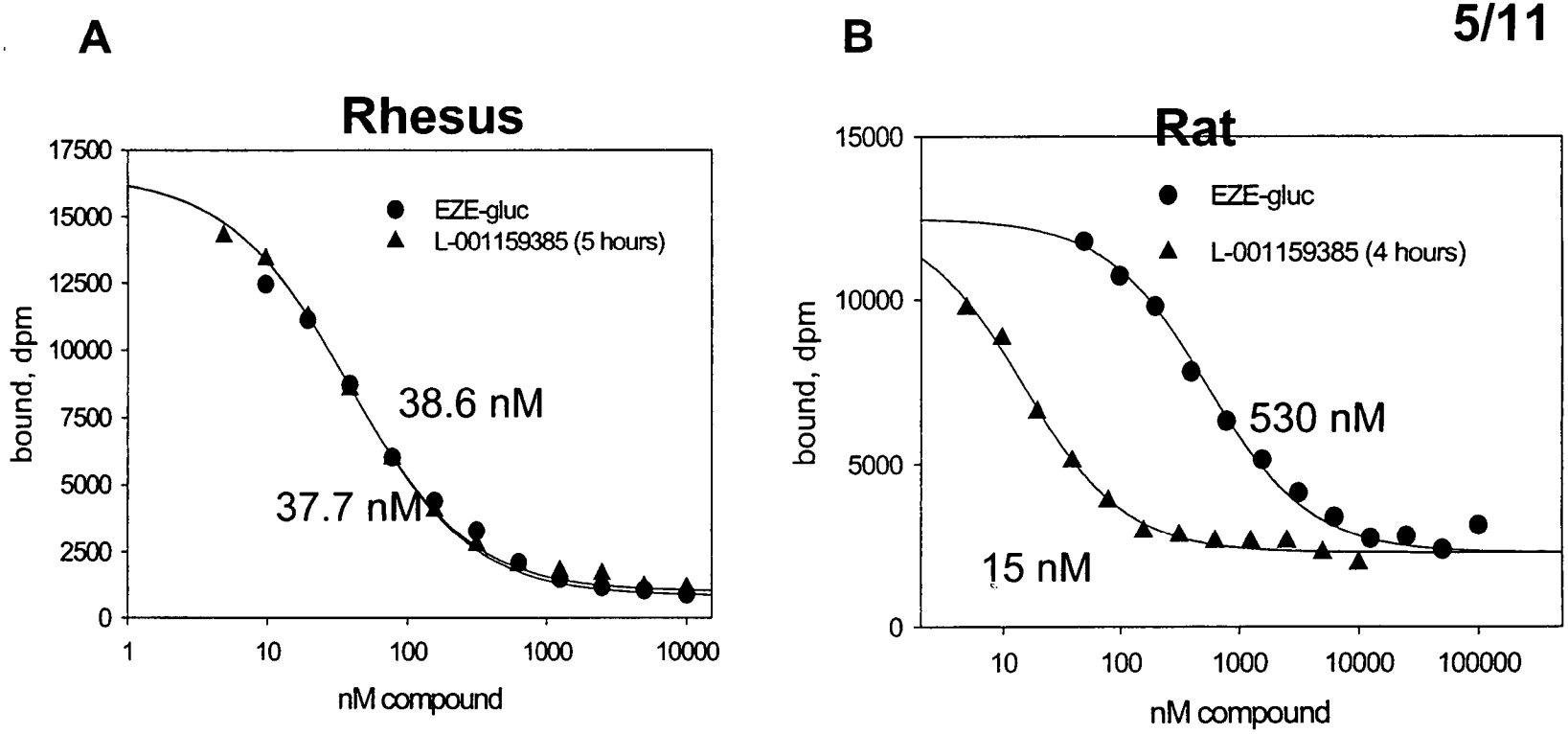


Figure 5. Displacement of 3H-EZE-glucuronide by EZE-glucuronide and L-00115938 in rhesus and rat BBMV.

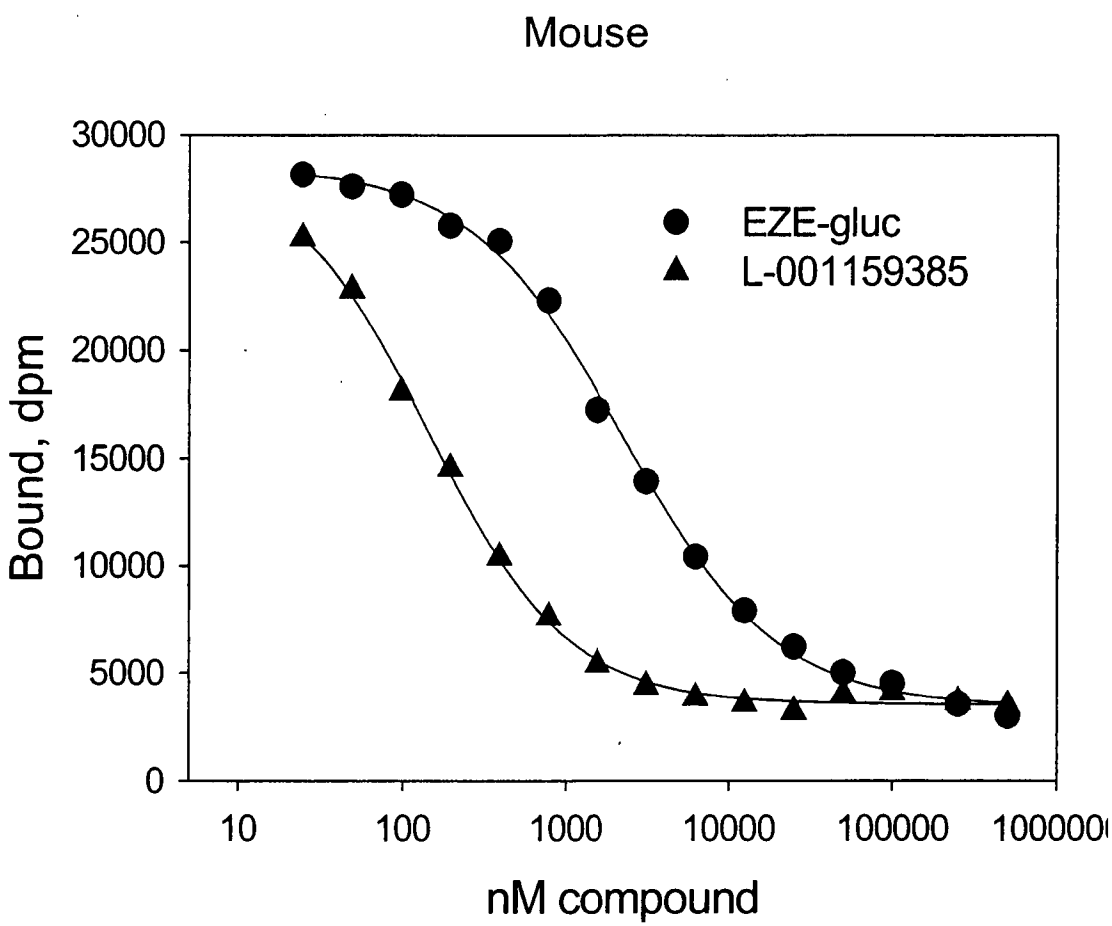


Figure 6. Displacement of ^{35}S-L-001159385 by EZE-glucuronide and L-001159385 in mouse BBMV.

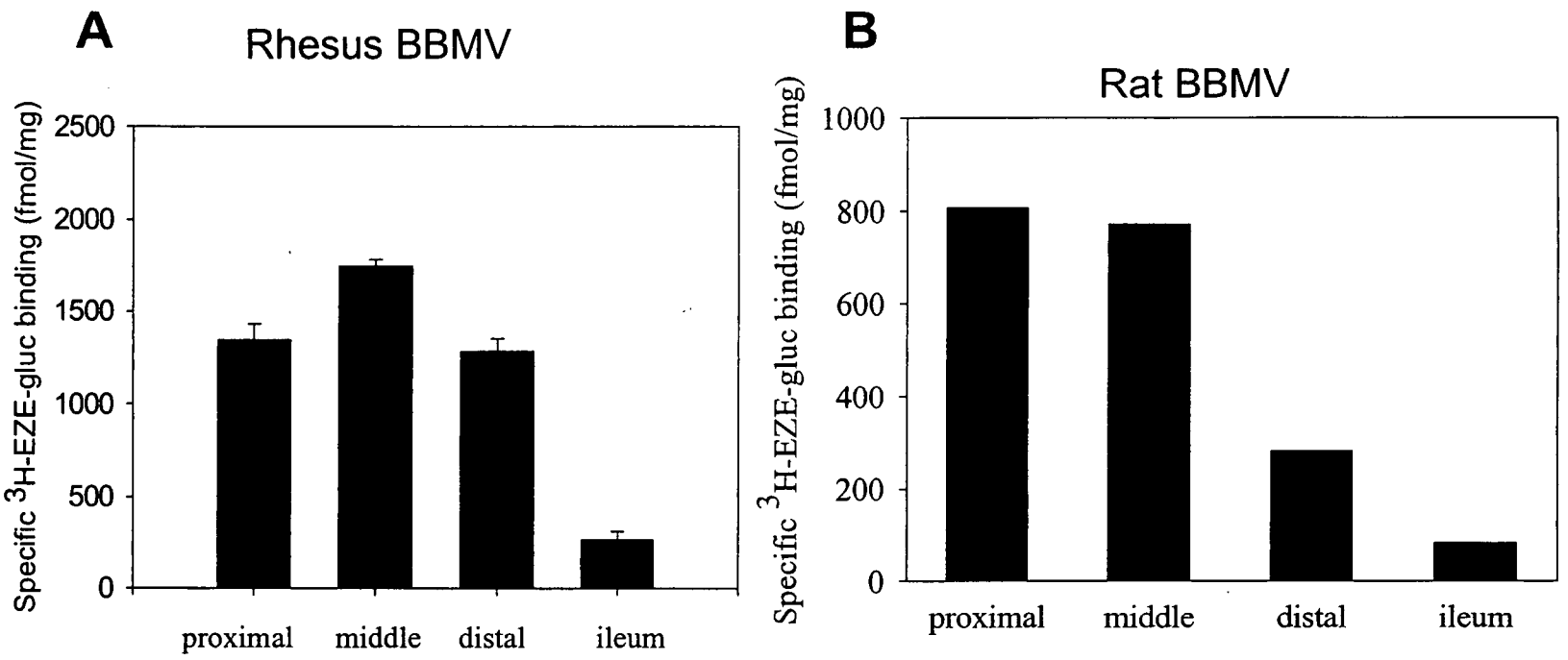
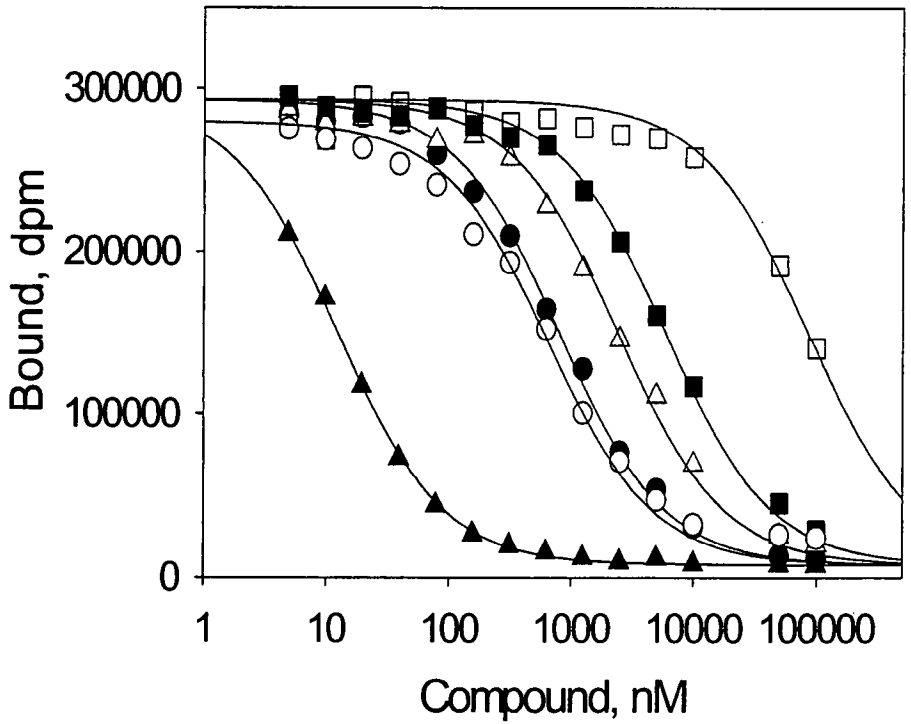
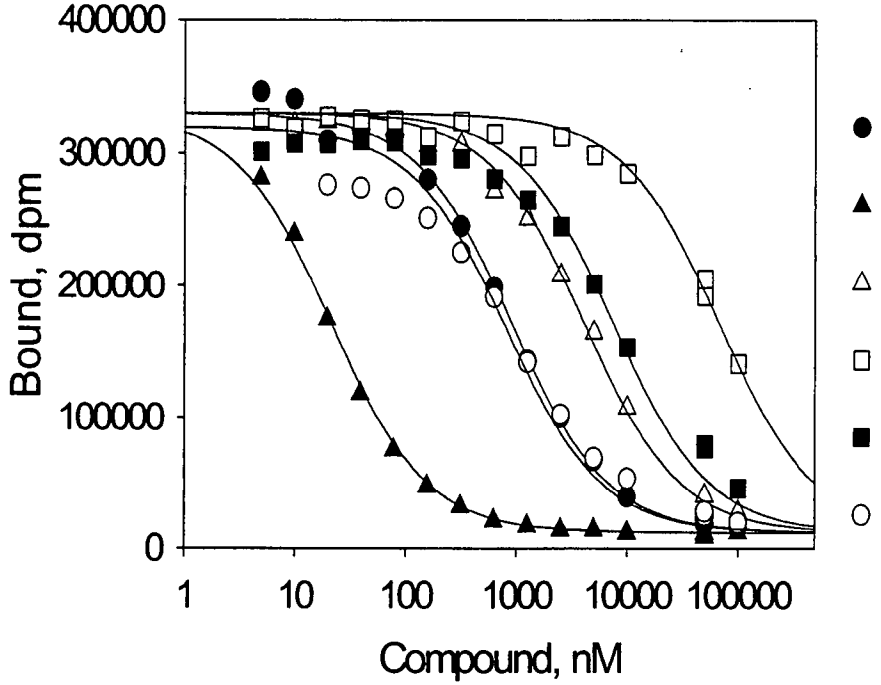


Figure 7. Intestinal distribution of ezetimibe binding sites.



		IC_{50}, nM
EZE-gluc	(●)	790
L-001159385	(▲)	12
L-001170530	(Δ)	2,400
L-001170533	( )	84,500
L-001170538	(■)	5,800
L-001176734	(○)	556

Figure 8. Displacement of ^{35}S-L-001159385 by EZE-glucuronide and analogs in transfected CHO cells expressing rat NPC1L1



	IC_{50}, nM
EZE-gluc	907
L-001159385	21
L-001170530	4,088
L-001170533	67,430
L-001170538	7,470
L-001176734	820

Figure 9. Displacement of ^{35}S-L-001159385 by EZE-glucuronide and analogs in transfected CHO cells expressing human NPC1L1

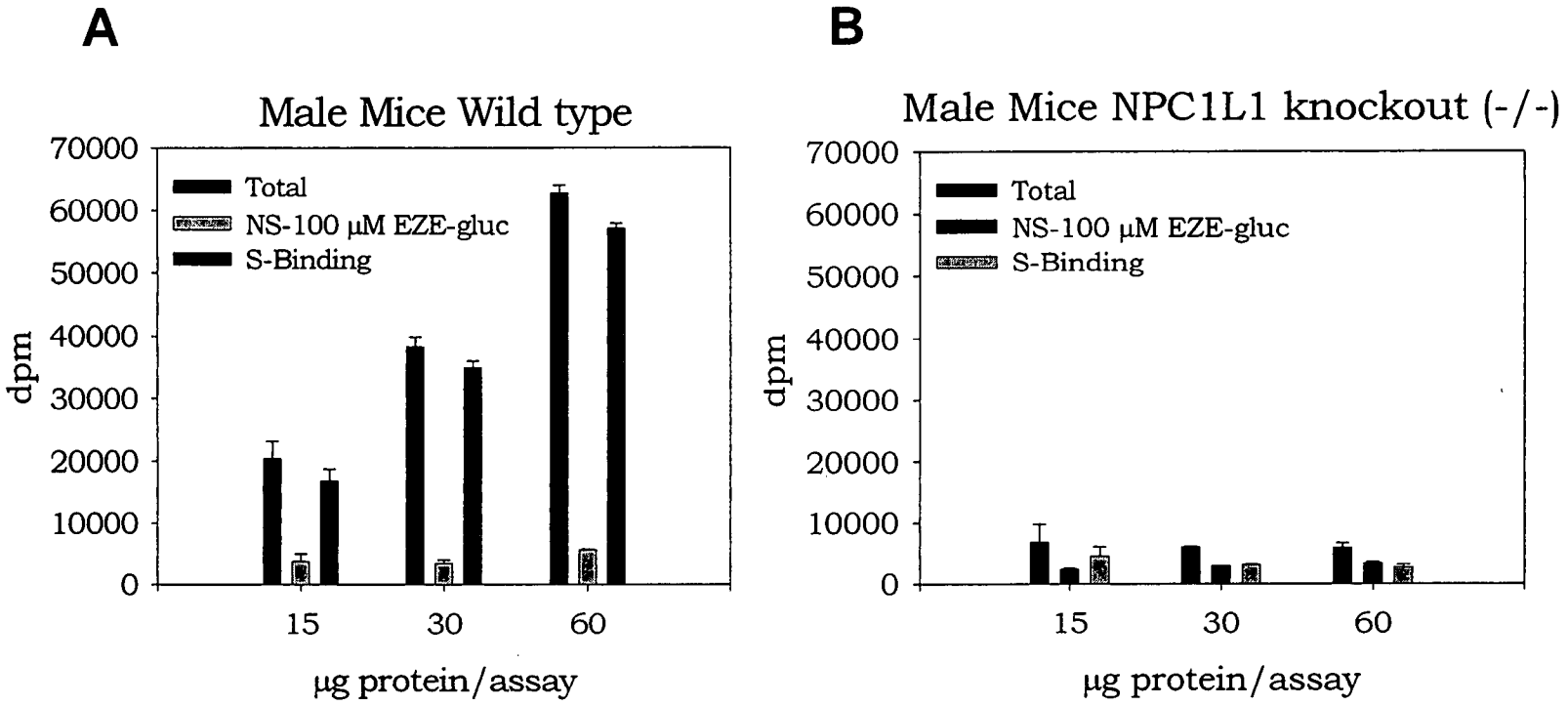


Figure 10. 35S-L-001159385 binding with brush border membranes from intestinal mucosal scrapings of male wild type (A) and NPC1L1 knockout (-/-) mice (B).

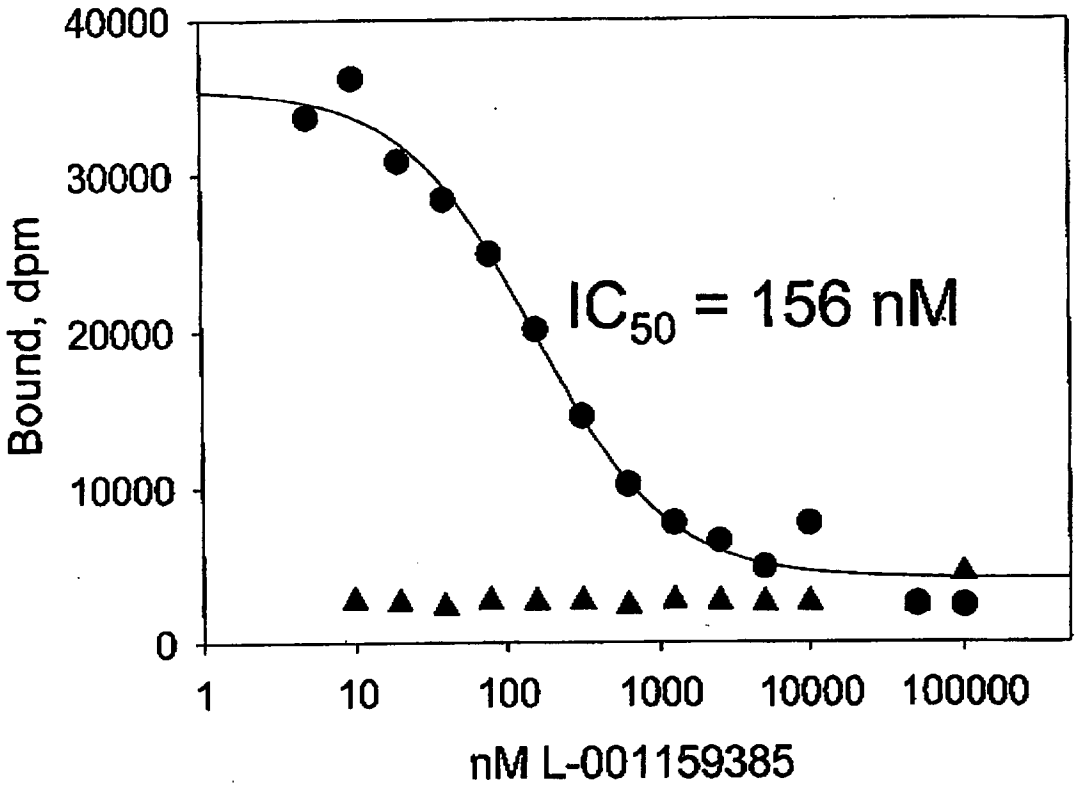


Figure 11. Displacement of [^{35}S]-L-001159385 by L-001159385 in mouse wild type and knockout mouse NPC1L1 (-/-) BBMV.
Aliquots (30 µg protein/assay) of male *mice BBMV from wild type (●)* and *knockout NPC1L1 (-/-) (▲) were incubated with 4 nM [^{35}S]-L-001159385 (~800,000 dpm/assay) at 37 °C in the presence of increasing concentrations of L-001159385 until equilibrium was achieved.*

SEQUENCE LISTING

<110> Garcia-Calvo, Margarita

<120> NPC1L1 (NPC3) AND METHODS OF IDENTIFYING LIGANDS THEREOF

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Ala Ala Gln Gly Glu Leu Tyr Thr Pro Lys His Glu Ala Gly Val Cys
            20                  25                  30

acc ttt tac gaa gag tgc ggg aaa aac cca gag ctc tct gga ggc ctc      144
Thr Phe Tyr Glu Glu Cys Gly Lys Asn Pro Glu Leu Ser Gly Gly Leu
        35                  40                  45

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65                  70                  75                  80

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Tyr Asn Gly Pro Asn Thr Thr Phe Ala Cys Cys Ser Thr Lys Gln Leu
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Leu Ser Leu Glu Ser Ser Met Ser Ile Thr Lys Ala Leu Leu Thr Arg
            100                 105                 110

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Cys Pro Ala Cys Ser Asp Asn Phe Val Ser Leu His Cys His Asn Thr
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Arg Gly Ala Gly Glu Pro Pro Ala Val Val Ala Tyr Glu Ala Phe Tyr
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Gln Arg Ser Phe Ala Glu Lys Ala Tyr Glu Ser Cys Ser Gln Val Arg
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Ile Pro Ala Ala Ala Ser Leu Ala Val Gly Ser Met Cys Gly Val Tyr
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225                 230                 235                 240

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Ala Pro Cys Asn Glu Ser Gln Gly Asp Asp Ser Ala Val Cys Ser Cys
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Gln Asp Cys Ala Ala Ser Cys Pro Val Ile Pro Pro Pro Glu Ala Leu
            260                 265                 270

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Arg Pro Ser Phe Tyr Met Gly Arg Met Pro Gly Trp Leu Ala Leu Ile
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Leu Arg Val Val Ser Asn Arg Asn Lys Asn Lys Ala Glu Gly Pro Gln
305                 310                 315                 320

gaa gcc ccc aaa ctc cct cat aag cac aaa ctc tca ccc cat acc atc     1008
Glu Ala Pro Lys Leu Pro His Lys His Lys Leu Ser Pro His Thr Ile
                325                 330                 335

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Leu Gly Arg Phe Phe Gln Asn Trp Gly Thr Arg Val Ala Ser Trp Pro
            340                 345                 350

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Leu Thr Val Leu Ala Leu Ser Phe Ile Val Val Ile Ala Leu Ala Ala
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gcc ccc aag agc cag gcc cgg aaa gag aag tct ttc cat gat gag cat     1200
Ala Pro Lys Ser Gln Ala Arg Lys Glu Lys Ser Phe His Asp Glu His
385                 390                 395                 400

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Phe Gly Pro Phe Phe Arg Thr Asn Gln Ile Phe Val Thr Ala Arg Asn
                405                 410                 415

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Arg Ser Ser Tyr Lys Tyr Asp Ser Leu Leu Leu Gly Ser Lys Asn Phe
            420                 425                 430

agt ggg atc ctg tcc ctg gac ttc ctg ctg gag ctg ctg gag ctt cag     1344
Ser Gly Ile Leu Ser Leu Asp Phe Leu Leu Glu Leu Leu Glu Leu Gln
        435                 440                 445

gag agg ctt cga cac ctg caa gtg tgg tcc cct gag gca gag cgc aac     1392
Glu Arg Leu Arg His Leu Gln Val Trp Ser Pro Glu Ala Glu Arg Asn
    450                 455                 460

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Ile Ser Leu Gln Asp Ile Cys Tyr Ala Pro Leu Asn Pro Tyr Asn Thr
465                 470                 475                 480

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Ser Leu Ser Asp Cys Cys Val Asn Ser Leu Leu Gln Tyr Phe Gln Asn
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Asp Tyr Gly Ala Pro Val Phe Pro Phe Leu Ala Val Gly Gly Tyr Gln
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Gly Thr Asp Tyr Ser Glu Ala Glu Ala Leu Ile Ile Thr Phe Ser Leu
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Ser Tyr Leu Ile Asp Tyr Phe Leu Phe Leu Asn Arg Tyr Leu Glu Val
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Arg Cys  Pro Lys Gly Gly Leu  Ala Ala Tyr Arg Thr  Ser Val Asn
    1025                1030                1035

ttg agc  tca gat ggc cag gtt  ata gcc tcc cag ttc  atg gcc tac      3159
Leu Ser  Ser Asp Gly Gln Val  Ile Ala Ser Gln Phe  Met Ala Tyr
    1040                1045                1050

cac aag  ccc tta agg aac tca  cag gac ttc aca gaa  gct ctc cgg      3204
His Lys  Pro Leu Arg Asn Ser  Gln Asp Phe Thr Glu  Ala Leu Arg
    1055                1060                1065

gcg tcc  cgg ttg cta gca gcc  aac atc aca gct gac  cta cgg aag      3249
Ala Ser  Arg Leu Leu Ala Ala  Asn Ile Thr Ala Asp  Leu Arg Lys
    1070                1075                1080

gtg cct  ggg aca gat cca aac  ttt gag gtc ttc cct  tac acg atc      3294
Val Pro  Gly Thr Asp Pro Asn  Phe Glu Val Phe Pro  Tyr Thr Ile
    1085                1090                1095

tcc aac  gtg ttc tac cag caa  tac ctg acg gtc ctt  cct gag gga      3339
Ser Asn  Val Phe Tyr Gln Gln  Tyr Leu Thr Val Leu  Pro Glu Gly
    1100                1105                1110

atc ttc  acc ctt gct ctt tgc  ttt gtg ccc acc ttt  gtt gtc tgc      3384
Ile Phe  Thr Leu Ala Leu Cys  Phe Val Pro Thr Phe  Val Val Cys
    1115                1120                1125

tac ctc  cta ctg ggc ctg gac  atg tgc tca ggg atc  ctc aac cta      3429
Tyr Leu  Leu Leu Gly Leu Asp  Met Cys Ser Gly Ile  Leu Asn Leu
    1130                1135                1140

ctc tcc  atc att atg att ctc  gtg gac acc att ggc  ctc atg gct      3474
Leu Ser  Ile Ile Met Ile Leu  Val Asp Thr Ile Gly  Leu Met Ala
    1145                1150                1155

gtg tgg  ggt atc agc tat aat  gcg gta tcc ctc atc  aac ctt gtc      3519
Val Trp  Gly Ile Ser Tyr Asn  Ala Val Ser Leu Ile  Asn Leu Val
    1160                1165                1170

acg gca  gtg ggc atg tct gtg  gag ttt gtg tcc cac  atc act cgg      3564
Thr Ala  Val Gly Met Ser Val  Glu Phe Val Ser His  Ile Thr Arg
    1175                1180                1185

tcc ttt  gct gta agc acc aag  cct acc cgg ctg gag  agg gct aaa      3609
Ser Phe  Ala Val Ser Thr Lys  Pro Thr Arg Leu Glu  Arg Ala Lys
    1190                1195                1200

gat gct  act gtc ttc atg ggc  agt gcg gtg ttt gct  gga gtg gcc      3654
Asp Ala  Thr Val Phe Met Gly  Ser Ala Val Phe Ala  Gly Val Ala
    1205                1210                1215

atg acc  aac ttc cca ggc atc  ctc atc ttg ggc ttt  gcc caa gcc      3699
```

```
Met Thr  Asn Phe Pro Gly Ile  Leu Ile Leu Gly Phe  Ala Gln Ala
    1220                 1225                 1230

cag ctt  att cag atc ttc ttc  ttc cgc ctc aac ctt  ctg atc acc      3744
Gln Leu  Ile Gln Ile Phe Phe  Phe Arg Leu Asn Leu  Leu Ile Thr
    1235                 1240                 1245

ttg ctg  ggt ctg ctg cat ggc  ctg gtc ttc ctg ccg  gtt gtc ctc      3789
Leu Leu  Gly Leu Leu His Gly  Leu Val Phe Leu Pro  Val Val Leu
    1250                 1255                 1260

agc tat  ctg gga cca gat gtt  aac caa gct ctg gta  cag gag gag      3834
Ser Tyr  Leu Gly Pro Asp Val  Asn Gln Ala Leu Val  Gln Glu Glu
    1265                 1270                 1275

aaa cta  gcc agc gag gca gca  gtg gcc cca gag cct  tct tgc cca      3879
Lys Leu  Ala Ser Glu Ala Ala  Val Ala Pro Glu Pro  Ser Cys Pro
    1280                 1285                 1290

cag tac  ccc tcc cct gct gat  gcg gat gcc aat gtt  aac tac ggc      3924
Gln Tyr  Pro Ser Pro Ala Asp  Ala Asp Ala Asn Val  Asn Tyr Gly
    1295                 1300                 1305

ttt gcc  cca gaa ctt gcc cac  gga gct aat gct gct  aga agc tct      3969
Phe Ala  Pro Glu Leu Ala His  Gly Ala Asn Ala Ala  Arg Ser Ser
    1310                 1315                 1320

ttg ccc  aaa agt gac caa aag  ttc taa                             3996
Leu Pro  Lys Ser Asp Gln Lys  Phe
    1325                 1330
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<210> 2

<211> 1331

<212> PRT

<213> Rattus sp.

<400> 2

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Met Ala Ala Ala Trp Leu Gly Trp Leu Leu Trp Ala Leu Leu Leu Ser
1               5                  10                  15

Ala Ala Gln Gly Glu Leu Tyr Thr Pro Lys His Glu Ala Gly Val Cys
            20                  25                  30

Thr Phe Tyr Glu Glu Cys Gly Lys Asn Pro Glu Leu Ser Gly Gly Leu
        35                  40                  45

Thr Ser Leu Ser Asn Val Ser Cys Leu Ser Asn Thr Pro Ala Arg His
    50                  55                  60
```

Val Thr Gly Glu His Leu Ala Leu Leu Gln Arg Ile Cys Pro Arg Leu
65 70 75 80

Tyr Asn Gly Pro Asn Thr Thr Phe Ala Cys Cys Ser Thr Lys Gln Leu
85 90 95

Leu Ser Leu Glu Ser Ser Met Ser Ile Thr Lys Ala Leu Leu Thr Arg
100 105 110

Cys Pro Ala Cys Ser Asp Asn Phe Val Ser Leu His Cys His Asn Thr
115 120 125

Cys Ser Pro Asp Gln Ser Leu Phe Ile Asn Val Thr Arg Val Val Glu
130 135 140

Arg Gly Ala Gly Glu Pro Pro Ala Val Val Ala Tyr Glu Ala Phe Tyr
145 150 155 160

Gln Arg Ser Phe Ala Glu Lys Ala Tyr Glu Ser Cys Ser Gln Val Arg
165 170 175

Ile Pro Ala Ala Ala Ser Leu Ala Val Gly Ser Met Cys Gly Val Tyr
180 185 190

Gly Ser Ala Leu Cys Asn Ala Gln Arg Trp Leu Asn Phe Gln Gly Asp
195 200 205

Thr Gly Asn Gly Leu Ala Pro Leu Asp Ile Thr Phe His Leu Leu Glu
210 215 220

Pro Gly Gln Ala Leu Pro Asp Gly Ile Gln Pro Leu Asn Gly Lys Ile
225 230 235 240

Ala Pro Cys Asn Glu Ser Gln Gly Asp Asp Ser Ala Val Cys Ser Cys
245 250 255

Gln Asp Cys Ala Ala Ser Cys Pro Val Ile Pro Pro Pro Glu Ala Leu
260 265 270

Arg Pro Ser Phe Tyr Met Gly Arg Met Pro Gly Trp Leu Ala Leu Ile
275 280 285

Ile Ile Phe Thr Ala Val Phe Val Leu Leu Ser Ala Val Leu Val Arg
290 295 300

```
Leu Arg Val Val Ser Asn Arg Asn Lys Asn Lys Ala Glu Gly Pro Gln
305                 310                 315                 320

Glu Ala Pro Lys Leu Pro His Lys His Lys Leu Ser Pro His Thr Ile
                325                 330                 335

Leu Gly Arg Phe Phe Gln Asn Trp Gly Thr Arg Val Ala Ser Trp Pro
            340                 345                 350

Leu Thr Val Leu Ala Leu Ser Phe Ile Val Val Ile Ala Leu Ala Ala
        355                 360                 365

Gly Leu Thr Phe Ile Glu Leu Thr Thr Asp Pro Val Glu Leu Trp Ser
    370                 375                 380

Ala Pro Lys Ser Gln Ala Arg Lys Glu Lys Ser Phe His Asp Glu His
385                 390                 395                 400

Phe Gly Pro Phe Phe Arg Thr Asn Gln Ile Phe Val Thr Ala Arg Asn
                405                 410                 415

Arg Ser Ser Tyr Lys Tyr Asp Ser Leu Leu Leu Gly Ser Lys Asn Phe
            420                 425                 430

Ser Gly Ile Leu Ser Leu Asp Phe Leu Leu Glu Leu Leu Glu Leu Gln
        435                 440                 445

Glu Arg Leu Arg His Leu Gln Val Trp Ser Pro Glu Ala Glu Arg Asn
    450                 455                 460

Ile Ser Leu Gln Asp Ile Cys Tyr Ala Pro Leu Asn Pro Tyr Asn Thr
465                 470                 475                 480

Ser Leu Ser Asp Cys Cys Val Asn Ser Leu Leu Gln Tyr Phe Gln Asn
                485                 490                 495

Asn Arg Thr Leu Leu Met Leu Thr Ala Asn Gln Thr Leu Asn Gly Gln
            500                 505                 510

Thr Ser Leu Val Asp Trp Lys Asp His Phe Leu Tyr Cys Ala Asn Ala
        515                 520                 525

Pro Leu Thr Phe Lys Asp Gly Thr Ser Leu Ala Leu Ser Cys Met Ala
```

```
    530                 535                 540

Asp Tyr Gly Ala Pro Val Phe Pro Phe Leu Ala Val Gly Gly Tyr Gln
545                 550                 555                 560

Gly Thr Asp Tyr Ser Glu Ala Glu Ala Leu Ile Ile Thr Phe Ser Leu
                565                 570                 575

Asn Asn Tyr Pro Ala Asp Asp Pro Arg Met Ala Gln Ala Lys Leu Trp
            580                 585                 590

Glu Glu Ala Phe Leu Lys Glu Met Glu Ser Phe Gln Arg Asn Thr Ser
        595                 600                 605

Asp Lys Phe Gln Val Ala Phe Ser Ala Glu Arg Ser Leu Glu Asp Glu
    610                 615                 620

Ile Asn Arg Thr Thr Ile Gln Asp Leu Pro Val Phe Ala Val Ser Tyr
625                 630                 635                 640

Ile Ile Val Phe Leu Tyr Ile Ser Leu Ala Leu Gly Ser Tyr Ser Arg
                645                 650                 655

Cys Ser Arg Val Ala Val Glu Ser Lys Ala Thr Leu Gly Leu Gly Gly
            660                 665                 670

Val Ile Val Val Leu Gly Ala Val Leu Ala Ala Met Gly Phe Tyr Ser
        675                 680                 685

Tyr Leu Gly Val Pro Ser Ser Leu Val Ile Ile Gln Val Val Pro Phe
    690                 695                 700

Leu Val Leu Ala Val Gly Ala Asp Asn Ile Phe Ile Phe Val Leu Glu
705                 710                 715                 720

Tyr Gln Arg Leu Pro Arg Met Pro Gly Glu Gln Arg Glu Ala His Ile
                725                 730                 735

Gly Arg Thr Leu Gly Ser Val Ala Pro Ser Met Leu Leu Cys Ser Leu
            740                 745                 750

Ser Glu Ala Ile Cys Phe Phe Leu Gly Ala Leu Thr Pro Met Pro Ala
        755                 760                 765
```

```
Val Arg Thr Phe Ala Leu Thr Ser Gly Leu Ala Ile Ile Leu Asp Phe
    770                 775                 780

Leu Leu Gln Met Thr Ala Phe Val Ala Leu Leu Ser Leu Asp Ser Lys
785                 790                 795                 800

Arg Gln Glu Ala Ser Arg Pro Asp Val Leu Cys Cys Phe Ser Thr Arg
                805                 810                 815

Lys Leu Pro Pro Pro Lys Glu Lys Glu Gly Leu Leu Leu Arg Phe Phe
            820                 825                 830

Arg Lys Ile Tyr Ala Pro Phe Leu Leu His Arg Phe Ile Arg Pro Val
        835                 840                 845

Val Met Leu Leu Phe Leu Thr Leu Phe Gly Ala Asn Leu Tyr Leu Met
    850                 855                 860

Cys Asn Ile Asn Val Gly Leu Asp Gln Glu Leu Ala Leu Pro Lys Asp
865                 870                 875                 880

Ser Tyr Leu Ile Asp Tyr Phe Leu Phe Leu Asn Arg Tyr Leu Glu Val
                885                 890                 895

Gly Pro Pro Val Tyr Phe Val Thr Thr Ser Gly Phe Asn Phe Ser Ser
            900                 905                 910

Glu Ala Gly Met Asn Ala Thr Cys Ser Ser Ala Gly Cys Lys Ser Phe
        915                 920                 925

Ser Leu Thr Gln Lys Ile Gln Tyr Ala Ser Glu Phe Pro Asp Gln Ser
    930                 935                 940

Tyr Val Ala Ile Ala Ala Ser Ser Trp Val Asp Asp Phe Ile Asp Trp
945                 950                 955                 960

Leu Thr Pro Ser Ser Ser Cys Cys Arg Leu Tyr Ile Arg Gly Pro His
                965                 970                 975

Lys Asp Glu Phe Cys Pro Ser Thr Asp Thr Ser Phe Asn Cys Leu Lys
            980                 985                 990

Asn Cys Met Asn Arg Thr Leu Gly  Pro Val Arg Pro Thr  Ala Glu Gln
        995                 1000                1005
```

Phe His Lys Tyr Leu Pro Trp Phe Leu Asn Asp Pro Pro Asn Ile
1010 1015 1020

Arg Cys Pro Lys Gly Gly Leu Ala Ala Tyr Arg Thr Ser Val Asn
1025 1030 1035

Leu Ser Ser Asp Gly Gln Val Ile Ala Ser Gln Phe Met Ala Tyr
1040 1045 1050

His Lys Pro Leu Arg Asn Ser Gln Asp Phe Thr Glu Ala Leu Arg
1055 1060 1065

Ala Ser Arg Leu Leu Ala Ala Asn Ile Thr Ala Asp Leu Arg Lys
1070 1075 1080

Val Pro Gly Thr Asp Pro Asn Phe Glu Val Phe Pro Tyr Thr Ile
1085 1090 1095

Ser Asn Val Phe Tyr Gln Gln Tyr Leu Thr Val Leu Pro Glu Gly
1100 1105 1110

Ile Phe Thr Leu Ala Leu Cys Phe Val Pro Thr Phe Val Val Cys
1115 1120 1125

Tyr Leu Leu Leu Gly Leu Asp Met Cys Ser Gly Ile Leu Asn Leu
1130 1135 1140

Leu Ser Ile Ile Met Ile Leu Val Asp Thr Ile Gly Leu Met Ala
1145 1150 1155

Val Trp Gly Ile Ser Tyr Asn Ala Val Ser Leu Ile Asn Leu Val
1160 1165 1170

Thr Ala Val Gly Met Ser Val Glu Phe Val Ser His Ile Thr Arg
1175 1180 1185

Ser Phe Ala Val Ser Thr Lys Pro Thr Arg Leu Glu Arg Ala Lys
1190 1195 1200

Asp Ala Thr Val Phe Met Gly Ser Ala Val Phe Ala Gly Val Ala
1205 1210 1215

Met Thr Asn Phe Pro Gly Ile Leu Ile Leu Gly Phe Ala Gln Ala
1220 1225 1230

```
Gln Leu  Ile Gln Ile Phe Phe  Phe Arg Leu Asn Leu  Leu Ile Thr
    1235                1240                1245

Leu Leu  Gly Leu Leu His Gly  Leu Val Phe Leu Pro  Val Val Leu
    1250                1255                1260

Ser Tyr  Leu Gly Pro Asp Val  Asn Gln Ala Leu Val  Gln Glu Glu
    1265                1270                1275

Lys Leu  Ala Ser Glu Ala Ala  Val Ala Pro Glu Pro  Ser Cys Pro
    1280                1285                1290

Gln Tyr  Pro Ser Pro Ala Asp  Ala Asp Ala Asn Val  Asn Tyr Gly
    1295                1300                1305

Phe Ala  Pro Glu Leu Ala His  Gly Ala Asn Ala Ala  Arg Ser Ser
    1310                1315                1320

Leu Pro  Lys Ser Asp Gln Lys  Phe
    1325                1330
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<210> 3

<211> 3999

<212> DNA

<213> Homo sapiens

<220>

<221> CDS

<222> (1)..(3999)

<223>

<400> 3

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atg gcg gag gcc ggc ctg agg ggc tgg ctg ctg tgg gcc ctg ctc ctg       48
Met Ala Glu Ala Gly Leu Arg Gly Trp Leu Leu Trp Ala Leu Leu Leu
1               5                   10                  15

cgc ttg gcc cag agt gag cct tac aca acc atc cac cag cct ggc tac       96
Arg Leu Ala Gln Ser Glu Pro Tyr Thr Thr Ile His Gln Pro Gly Tyr
            20                  25                  30
```

```
tgc gcc ttc tat gac gaa tgt ggg aag aac cca gag ctg tct gga agc      144
Cys Ala Phe Tyr Asp Glu Cys Gly Lys Asn Pro Glu Leu Ser Gly Ser
        35                  40                  45

ctc atg aca ctc tcc aac gtg tcc tgc ctg tcc aac acg ccg gcc cgc      192
Leu Met Thr Leu Ser Asn Val Ser Cys Leu Ser Asn Thr Pro Ala Arg
    50                  55                  60

aag atc aca ggt gat cac ctg atc cta tta cag aag atc tgc ccc cgc      240
Lys Ile Thr Gly Asp His Leu Ile Leu Leu Gln Lys Ile Cys Pro Arg
65                  70                  75                  80

ctc tac acc ggc ccc aac acc caa gcc tgc tgc tcc gcc aag cag ctg      288
Leu Tyr Thr Gly Pro Asn Thr Gln Ala Cys Cys Ser Ala Lys Gln Leu
                85                  90                  95

gta tca ctg gaa gcg agt ctg tcg atc acc aag gcc ctc ctc acc cgc      336
Val Ser Leu Glu Ala Ser Leu Ser Ile Thr Lys Ala Leu Leu Thr Arg
            100                 105                 110

tgc cca gcc tgc tct gac aat ttt gtg aac ctg cac tgc cac aac acg      384
Cys Pro Ala Cys Ser Asp Asn Phe Val Asn Leu His Cys His Asn Thr
        115                 120                 125

tgc agc ccc aat cag agc ctc ttc atc aat gtg acc cgc gtg gcc cag      432
Cys Ser Pro Asn Gln Ser Leu Phe Ile Asn Val Thr Arg Val Ala Gln
    130                 135                 140

cta ggg gct gga caa ctc cca gct gtg gtg gcc tat gag gcc ttc tac      480
Leu Gly Ala Gly Gln Leu Pro Ala Val Val Ala Tyr Glu Ala Phe Tyr
145                 150                 155                 160

cag cat agc ttt gcc gag cag agc tat gac tcc tgc agc cgt gtg cgc      528
Gln His Ser Phe Ala Glu Gln Ser Tyr Asp Ser Cys Ser Arg Val Arg
                165                 170                 175

gtc cct gca gct gcc acg ctg gct gtg ggc acc atg tgt ggc gtg tat      576
Val Pro Ala Ala Ala Thr Leu Ala Val Gly Thr Met Cys Gly Val Tyr
            180                 185                 190

ggc tct gcc ctt tgc aat gcc cag cgc tgg ctc aac ttc cag gga gac      624
Gly Ser Ala Leu Cys Asn Ala Gln Arg Trp Leu Asn Phe Gln Gly Asp
        195                 200                 205

aca ggc aat ggt ctg gcc cca ctg gac atc acc ttc cac ctc ttg gag      672
Thr Gly Asn Gly Leu Ala Pro Leu Asp Ile Thr Phe His Leu Leu Glu
    210                 215                 220

cct ggc cag gcc gtg ggg agt ggg att cag cct ctg aat gag ggg gtt      720
Pro Gly Gln Ala Val Gly Ser Gly Ile Gln Pro Leu Asn Glu Gly Val
225                 230                 235                 240

gca cgt tgc aat gag tcc caa ggt gac gac gtg gcg acc tgc tcc tgc      768
Ala Arg Cys Asn Glu Ser Gln Gly Asp Asp Val Ala Thr Cys Ser Cys
                245                 250                 255

caa gac tgt gct gca tcc tgt cct gcc ata gcc cgc ccc cag gcc ctc      816
Gln Asp Cys Ala Ala Ser Cys Pro Ala Ile Ala Arg Pro Gln Ala Leu
            260                 265                 270
```

```
gac tcc acc ttc tac ctg ggc cag atg ccg ggc agt ctg gtc ctc atc      864
Asp Ser Thr Phe Tyr Leu Gly Gln Met Pro Gly Ser Leu Val Leu Ile
        275                 280                 285

atc atc ctc tgc tct gtc ttc gct gtg gtc acc atc ctg ctt gtg gga      912
Ile Ile Leu Cys Ser Val Phe Ala Val Val Thr Ile Leu Leu Val Gly
    290                 295                 300

ttc cgt gtg gcc ccc gcc agg gac aaa agc aag atg gtg gac ccc aag      960
Phe Arg Val Ala Pro Ala Arg Asp Lys Ser Lys Met Val Asp Pro Lys
305                 310                 315                 320

aag ggc acc agc ctc tct gac aag ctc agc ttc tcc acc cac acc ctc     1008
Lys Gly Thr Ser Leu Ser Asp Lys Leu Ser Phe Ser Thr His Thr Leu
                325                 330                 335

ctt ggc cag ttc ttc cag ggc tgg ggc acg tgg gtg gct tcg tgg cct     1056
Leu Gly Gln Phe Phe Gln Gly Trp Gly Thr Trp Val Ala Ser Trp Pro
            340                 345                 350

ctg acc atc ttg gtg cta tct gtc atc ccg gtg gtg gcc ttg gca gcg     1104
Leu Thr Ile Leu Val Leu Ser Val Ile Pro Val Val Ala Leu Ala Ala
        355                 360                 365

ggc ctg gtc ttt aca gaa ctc act acg gac ccc gtg gag ctg tgg tcg     1152
Gly Leu Val Phe Thr Glu Leu Thr Thr Asp Pro Val Glu Leu Trp Ser
    370                 375                 380

gcc ccc aac agc caa gcc cgg agt gag aaa gct ttc cat gac cag cat     1200
Ala Pro Asn Ser Gln Ala Arg Ser Glu Lys Ala Phe His Asp Gln His
385                 390                 395                 400

ttc ggc ccc ttc ttc cga acc aac cag gtg atc ctg acg gct cct aac     1248
Phe Gly Pro Phe Phe Arg Thr Asn Gln Val Ile Leu Thr Ala Pro Asn
                405                 410                 415

cgg tcc agc tac agg tat gac tct ctg ctg ctg ggg ccc aag aac ttc     1296
Arg Ser Ser Tyr Arg Tyr Asp Ser Leu Leu Leu Gly Pro Lys Asn Phe
            420                 425                 430

agc gga atc ctg gac ctg gac ttg ctg ctg gag ctg cta gag ctg cag     1344
Ser Gly Ile Leu Asp Leu Asp Leu Leu Leu Glu Leu Leu Glu Leu Gln
        435                 440                 445

gag agg ctg cgg cac ctc cag gta tgg tcg ccc gaa gca cag cgc aac     1392
Glu Arg Leu Arg His Leu Gln Val Trp Ser Pro Glu Ala Gln Arg Asn
    450                 455                 460

atc tcc ctg cag gac atc tgc tac gcc ccc ctc aat ccg gac aat acc     1440
Ile Ser Leu Gln Asp Ile Cys Tyr Ala Pro Leu Asn Pro Asp Asn Thr
465                 470                 475                 480

agt ctc tac gac tgc tgc atc aac agc ctc ctg cag tat ttc cag aac     1488
Ser Leu Tyr Asp Cys Cys Ile Asn Ser Leu Leu Gln Tyr Phe Gln Asn
                485                 490                 495

aac cgc acg ctc ctg ctg ctc aca gcc aac cag aca ctg atg ggg cag     1536
Asn Arg Thr Leu Leu Leu Leu Thr Ala Asn Gln Thr Leu Met Gly Gln
```

```
            500                 505                 510

acc tcc caa gtc gac tgg aag gac cat ttt ctg tac tgt gcc aat gcc     1584
Thr Ser Gln Val Asp Trp Lys Asp His Phe Leu Tyr Cys Ala Asn Ala
        515                 520                 525

ccg ctc acc ttc aag gat ggc aca gcc ctg gcc ctg agc tgc atg gct     1632
Pro Leu Thr Phe Lys Asp Gly Thr Ala Leu Ala Leu Ser Cys Met Ala
    530                 535                 540

gac tac ggg gcc cct gtc ttc ccc ttc ctt gcc att ggg ggg tac aaa     1680
Asp Tyr Gly Ala Pro Val Phe Pro Phe Leu Ala Ile Gly Gly Tyr Lys
545                 550                 555                 560

gga aag gac tat tct gag gca gag gcc ctg atc atg acg ttc tcc ctc     1728
Gly Lys Asp Tyr Ser Glu Ala Glu Ala Leu Ile Met Thr Phe Ser Leu
                565                 570                 575

aac aat tac cct gcc ggg gac ccc cgt ctg gcc cag gcc aag ctg tgg     1776
Asn Asn Tyr Pro Ala Gly Asp Pro Arg Leu Ala Gln Ala Lys Leu Trp
            580                 585                 590

gag gag gcc ttc tta gag gaa atg cga gcc ttc cag cgt cgg atg gct     1824
Glu Glu Ala Phe Leu Glu Glu Met Arg Ala Phe Gln Arg Arg Met Ala
        595                 600                 605

ggc atg ttc cag gtc acg ttc acg gct gag cgc tct ctg gaa gac gag     1872
Gly Met Phe Gln Val Thr Phe Thr Ala Glu Arg Ser Leu Glu Asp Glu
    610                 615                 620

atc aat cgc acc aca gct gaa gac ctg ccc atc ttt gcc acc agc tac     1920
Ile Asn Arg Thr Thr Ala Glu Asp Leu Pro Ile Phe Ala Thr Ser Tyr
625                 630                 635                 640

att gtc ata ttc ctg tac atc tct ctg gcc ctg ggc agc tat tcc agc     1968
Ile Val Ile Phe Leu Tyr Ile Ser Leu Ala Leu Gly Ser Tyr Ser Ser
                645                 650                 655

tgg agc cga gtg atg gtg gac tcc aag gcc acg ctg ggc ctc ggc ggg     2016
Trp Ser Arg Val Met Val Asp Ser Lys Ala Thr Leu Gly Leu Gly Gly
            660                 665                 670

gtg gcc gtg gtc ctg gga gca gtc atg gct gcc atg ggc ttc ttc tcc     2064
Val Ala Val Val Leu Gly Ala Val Met Ala Ala Met Gly Phe Phe Ser
        675                 680                 685

tac ttg ggt atc cgc tcc tcc ctg gtc atc ctg caa gtg gtt cct ttc     2112
Tyr Leu Gly Ile Arg Ser Ser Leu Val Ile Leu Gln Val Val Pro Phe
    690                 695                 700

ctg gtg ctg tcc gtg ggg gct gat aac atc ttc atc ttt gtt ctc gag     2160
Leu Val Leu Ser Val Gly Ala Asp Asn Ile Phe Ile Phe Val Leu Glu
705                 710                 715                 720

tac cag agg ctg ccc cgg agg cct ggg gag cca cga gag gtc cac att     2208
Tyr Gln Arg Leu Pro Arg Arg Pro Gly Glu Pro Arg Glu Val His Ile
                725                 730                 735

ggg cga gcc cta ggc agg gtg gct ccc agc atg ctg ttg tgc agc ctc     2256
```

```
Gly Arg Ala Leu Gly Arg Val Ala Pro Ser Met Leu Leu Cys Ser Leu
            740                 745                 750

tct gag gcc atc tgc ttc ttc cta ggg gcc ctg acc ccc atg cca gct     2304
Ser Glu Ala Ile Cys Phe Phe Leu Gly Ala Leu Thr Pro Met Pro Ala
        755                 760                 765

gtg cgg acc ttt gcc ctg acc tct ggc ctt gca gtg atc ctt gac ttc     2352
Val Arg Thr Phe Ala Leu Thr Ser Gly Leu Ala Val Ile Leu Asp Phe
    770                 775                 780

ctc ctg cag atg tca gcc ttt gtg gcc ctg ctc tcc ctg gac agc aag     2400
Leu Leu Gln Met Ser Ala Phe Val Ala Leu Leu Ser Leu Asp Ser Lys
785                 790                 795                 800

agg cag gag gcc tcc cgg ttg gac gtc tgc tgc tgt gtc aag ccc cag     2448
Arg Gln Glu Ala Ser Arg Leu Asp Val Cys Cys Cys Val Lys Pro Gln
                805                 810                 815

gag ctg ccc ccg cct ggc cag gga gag ggg ctc ctg ctt ggc ttc ttc     2496
Glu Leu Pro Pro Pro Gly Gln Gly Glu Gly Leu Leu Leu Gly Phe Phe
            820                 825                 830

caa aag gct tat gcc ccc ttc ctg ctg cac tgg atc act cga ggt gtt     2544
Gln Lys Ala Tyr Ala Pro Phe Leu Leu His Trp Ile Thr Arg Gly Val
        835                 840                 845

gtg ctg ctg ctg ttt ctc gcc ctg ttc gga gtg agc ctc tac tcc atg     2592
Val Leu Leu Leu Phe Leu Ala Leu Phe Gly Val Ser Leu Tyr Ser Met
    850                 855                 860

tgc cac atc agc gtg gga ctg gac cag gag ctg gcc ctg ccc aag gac     2640
Cys His Ile Ser Val Gly Leu Asp Gln Glu Leu Ala Leu Pro Lys Asp
865                 870                 875                 880

tcg tac ctg ctt gac tat ttc ctc ttt ctg aac cgc tac ttc gag gtg     2688
Ser Tyr Leu Leu Asp Tyr Phe Leu Phe Leu Asn Arg Tyr Phe Glu Val
                885                 890                 895

ggg gcc ccg gtg tac ttt gtt acc acc ttg ggc tac aac ttc tcc agc     2736
Gly Ala Pro Val Tyr Phe Val Thr Thr Leu Gly Tyr Asn Phe Ser Ser
            900                 905                 910

gag gct ggg atg aat gcc atc tgc tcc agt gca ggc tgc aac aac ttc     2784
Glu Ala Gly Met Asn Ala Ile Cys Ser Ser Ala Gly Cys Asn Asn Phe
        915                 920                 925

tcc ttc acc cag aag atc cag tat gcc aca gag ttc cct gag cag tct     2832
Ser Phe Thr Gln Lys Ile Gln Tyr Ala Thr Glu Phe Pro Glu Gln Ser
    930                 935                 940

tac ctg gcc atc cct gcc tcc tcc tgg gtg gat gac ttc att gac tgg     2880
Tyr Leu Ala Ile Pro Ala Ser Ser Trp Val Asp Asp Phe Ile Asp Trp
945                 950                 955                 960

ctg acc ccg tcc tcc tgc tgc cgc ctt tat ata tct ggc ccc aat aag     2928
Leu Thr Pro Ser Ser Cys Cys Arg Leu Tyr Ile Ser Gly Pro Asn Lys
                965                 970                 975
```

```
gac aag ttc tgc ccc tcg acc gtc aac tct ctg aac tgc cta aag aac     2976
Asp Lys Phe Cys Pro Ser Thr Val Asn Ser Leu Asn Cys Leu Lys Asn
            980                 985                 990

tgc atg agc atc acg atg ggc tct  gtg agg ccc tcg gtg  gag cag ttc   3024
Cys Met Ser Ile Thr Met Gly Ser  Val Arg Pro Ser Val  Glu Gln Phe
        995                1000                1005

cat aag  tat ctt ccc tgg ttc  ctg aac gac cgg ccc  aac atc aaa      3069
His Lys  Tyr Leu Pro Trp Phe  Leu Asn Asp Arg Pro  Asn Ile Lys
    1010                1015                1020

tgt ccc  aaa ggc ggc ctg gca  gca tac agc acc tct  gtg aac ttg      3114
Cys Pro  Lys Gly Gly Leu Ala  Ala Tyr Ser Thr Ser  Val Asn Leu
    1025                1030                1035

act tca  gat ggc cag gtt tta  gcc tcc agg ttc atg  gcc tat cac      3159
Thr Ser  Asp Gly Gln Val Leu  Ala Ser Arg Phe Met  Ala Tyr His
    1040                1045                1050

aag ccc  ctg aaa aac tca cag  gat tac aca gaa gct  ctg cgg gca      3204
Lys Pro  Leu Lys Asn Ser Gln  Asp Tyr Thr Glu Ala  Leu Arg Ala
    1055                1060                1065

gct cga  gag ctg gca gcc aac  atc act gct gac ctg  cgg aaa gtg      3249
Ala Arg  Glu Leu Ala Ala Asn  Ile Thr Ala Asp Leu  Arg Lys Val
    1070                1075                1080

cct gga  aca gac ccg gct ttt  gag gtc ttc ccc tac  acg atc acc      3294
Pro Gly  Thr Asp Pro Ala Phe  Glu Val Phe Pro Tyr  Thr Ile Thr
    1085                1090                1095

aat gtg  ttt tat gag cag tac  ctg acc atc ctc cct  gag ggg ctc      3339
Asn Val  Phe Tyr Glu Gln Tyr  Leu Thr Ile Leu Pro  Glu Gly Leu
    1100                1105                1110

ttc atg  ctc agc ctc tgc ctt  gtg ccc acc ttc gct  gtc tcc tgc      3384
Phe Met  Leu Ser Leu Cys Leu  Val Pro Thr Phe Ala  Val Ser Cys
    1115                1120                1125

ctc ctg  ctg ggc ctg gac ctg  cgc tcc ggc ctc ctc  aac ctg ctc      3429
Leu Leu  Leu Gly Leu Asp Leu  Arg Ser Gly Leu Leu  Asn Leu Leu
    1130                1135                1140

tcc att  gtc atg atc ctc gtg  gac act gtc ggc ttc  atg gcc ctg      3474
Ser Ile  Val Met Ile Leu Val  Asp Thr Val Gly Phe  Met Ala Leu
    1145                1150                1155

tgg gac  atc agt tac aat gct  gtg tcc ctc atc aac  ctg gtc tcg      3519
Trp Asp  Ile Ser Tyr Asn Ala  Val Ser Leu Ile Asn  Leu Val Ser
    1160                1165                1170

gcg gtg  ggc atg tct gtg gag  ttt gtg tcc cac att  acc cgc tcc      3564
Ala Val  Gly Met Ser Val Glu  Phe Val Ser His Ile  Thr Arg Ser
    1175                1180                1185

ttt gcc  atc agc acc aag ccc  acc tgg ctg gag agg  gcc aaa gag      3609
Phe Ala  Ile Ser Thr Lys Pro  Thr Trp Leu Glu Arg  Ala Lys Glu
    1190                1195                1200
```

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gcc acc  atc tct atg gga agt  gcg gtg ttt gca ggt  gtg gcc atg      3654
Ala Thr  Ile Ser Met Gly Ser  Ala Val Phe Ala Gly  Val Ala Met
    1205                 1210                 1215

acc aac  ctg cct ggc atc ctt  gtc ctg ggc ctc gcc  aag gcc cag      3699
Thr Asn  Leu Pro Gly Ile Leu  Val Leu Gly Leu Ala  Lys Ala Gln
    1220                 1225                 1230

ctc att  cag atc ttc ttc ttc  cgc ctc aac ctc ctg  atc act ctg      3744
Leu Ile  Gln Ile Phe Phe Phe  Arg Leu Asn Leu Leu  Ile Thr Leu
    1235                 1240                 1245

ctg ggc  ctg ctg cat ggc ttg  gtc ttc ctg ccc gtc  atc ctc agc      3789
Leu Gly  Leu Leu His Gly Leu  Val Phe Leu Pro Val  Ile Leu Ser
    1250                 1255                 1260

tac gtg  ggg cct gac gtt aac  ccg gct ctg gca ctg  gag cag aag      3834
Tyr Val  Gly Pro Asp Val Asn  Pro Ala Leu Ala Leu  Glu Gln Lys
    1265                 1270                 1275

cgg gct  gag gag gcg gtg gca  gca gtc atg gtg gcc  tct tgc cca      3879
Arg Ala  Glu Glu Ala Val Ala  Ala Val Met Val Ala  Ser Cys Pro
    1280                 1285                 1290

aat cac  ccc tcc cga gtc tcc  aca gct gac aac atc  tat gtc aac      3924
Asn His  Pro Ser Arg Val Ser  Thr Ala Asp Asn Ile  Tyr Val Asn
    1295                 1300                 1305

cac agc  ttt gaa ggt tct atc  aaa ggt gct ggt gcc  atc agc aac      3969
His Ser  Phe Glu Gly Ser Ile  Lys Gly Ala Gly Ala  Ile Ser Asn
    1310                 1315                 1320

ttc ttg  ccc aac aat ggg cgg  cag ttc tga                          3999
Phe Leu  Pro Asn Asn Gly Arg  Gln Phe
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<211> 1332

<212> PRT

<213> Homo sapiens

<400> 4

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Arg Leu Ala Gln Ser Glu Pro Tyr Thr Thr Ile His Gln Pro Gly Tyr
            20                  25                  30

Cys Ala Phe Tyr Asp Glu Cys Gly Lys Asn Pro Glu Leu Ser Gly Ser
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```
        35                  40                  45

Leu Met Thr Leu Ser Asn Val Ser Cys Leu Ser Asn Thr Pro Ala Arg
    50                  55                  60

Lys Ile Thr Gly Asp His Leu Ile Leu Leu Gln Lys Ile Cys Pro Arg
65                  70                  75                  80

Leu Tyr Thr Gly Pro Asn Thr Gln Ala Cys Cys Ser Ala Lys Gln Leu
                85                  90                  95

Val Ser Leu Glu Ala Ser Leu Ser Ile Thr Lys Ala Leu Leu Thr Arg
            100                 105                 110

Cys Pro Ala Cys Ser Asp Asn Phe Val Asn Leu His Cys His Asn Thr
        115                 120                 125

Cys Ser Pro Asn Gln Ser Leu Phe Ile Asn Val Thr Arg Val Ala Gln
    130                 135                 140

Leu Gly Ala Gly Gln Leu Pro Ala Val Val Ala Tyr Glu Ala Phe Tyr
145                 150                 155                 160

Gln His Ser Phe Ala Glu Gln Ser Tyr Asp Ser Cys Ser Arg Val Arg
                165                 170                 175

Val Pro Ala Ala Ala Thr Leu Ala Val Gly Thr Met Cys Gly Val Tyr
            180                 185                 190

Gly Ser Ala Leu Cys Asn Ala Gln Arg Trp Leu Asn Phe Gln Gly Asp
        195                 200                 205

Thr Gly Asn Gly Leu Ala Pro Leu Asp Ile Thr Phe His Leu Leu Glu
    210                 215                 220

Pro Gly Gln Ala Val Gly Ser Gly Ile Gln Pro Leu Asn Glu Gly Val
225                 230                 235                 240

Ala Arg Cys Asn Glu Ser Gln Gly Asp Asp Val Ala Thr Cys Ser Cys
                245                 250                 255

Gln Asp Cys Ala Ala Ser Cys Pro Ala Ile Ala Arg Pro Gln Ala Leu
            260                 265                 270
```

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Asp Ser Thr Phe Tyr Leu Gly Gln Met Pro Gly Ser Leu Val Leu Ile
        275                 280                 285

Ile Ile Leu Cys Ser Val Phe Ala Val Val Thr Ile Leu Leu Val Gly
    290                 295                 300

Phe Arg Val Ala Pro Ala Arg Asp Lys Ser Lys Met Val Asp Pro Lys
305                 310                 315                 320

Lys Gly Thr Ser Leu Ser Asp Lys Leu Ser Phe Ser Thr His Thr Leu
                325                 330                 335

Leu Gly Gln Phe Phe Gln Gly Trp Gly Thr Trp Val Ala Ser Trp Pro
            340                 345                 350

Leu Thr Ile Leu Val Leu Ser Val Ile Pro Val Val Ala Leu Ala Ala
        355                 360                 365

Gly Leu Val Phe Thr Glu Leu Thr Thr Asp Pro Val Glu Leu Trp Ser
    370                 375                 380

Ala Pro Asn Ser Gln Ala Arg Ser Glu Lys Ala Phe His Asp Gln His
385                 390                 395                 400

Phe Gly Pro Phe Phe Arg Thr Asn Gln Val Ile Leu Thr Ala Pro Asn
                405                 410                 415

Arg Ser Ser Tyr Arg Tyr Asp Ser Leu Leu Leu Gly Pro Lys Asn Phe
            420                 425                 430

Ser Gly Ile Leu Asp Leu Asp Leu Leu Leu Glu Leu Leu Glu Leu Gln
        435                 440                 445

Glu Arg Leu Arg His Leu Gln Val Trp Ser Pro Glu Ala Gln Arg Asn
    450                 455                 460

Ile Ser Leu Gln Asp Ile Cys Tyr Ala Pro Leu Asn Pro Asp Asn Thr
465                 470                 475                 480

Ser Leu Tyr Asp Cys Cys Ile Asn Ser Leu Leu Gln Tyr Phe Gln Asn
                485                 490                 495

Asn Arg Thr Leu Leu Leu Leu Thr Ala Asn Gln Thr Leu Met Gly Gln
            500                 505                 510
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```
Thr Ser Gln Val Asp Trp Lys Asp His Phe Leu Tyr Cys Ala Asn Ala
        515                 520                 525

Pro Leu Thr Phe Lys Asp Gly Thr Ala Leu Ala Leu Ser Cys Met Ala
    530                 535                 540

Asp Tyr Gly Ala Pro Val Phe Pro Phe Leu Ala Ile Gly Gly Tyr Lys
545                 550                 555                 560

Gly Lys Asp Tyr Ser Glu Ala Glu Ala Leu Ile Met Thr Phe Ser Leu
                565                 570                 575

Asn Asn Tyr Pro Ala Gly Asp Pro Arg Leu Ala Gln Ala Lys Leu Trp
            580                 585                 590

Glu Glu Ala Phe Leu Glu Glu Met Arg Ala Phe Gln Arg Arg Met Ala
        595                 600                 605

Gly Met Phe Gln Val Thr Phe Thr Ala Glu Arg Ser Leu Glu Asp Glu
    610                 615                 620

Ile Asn Arg Thr Thr Ala Glu Asp Leu Pro Ile Phe Ala Thr Ser Tyr
625                 630                 635                 640

Ile Val Ile Phe Leu Tyr Ile Ser Leu Ala Leu Gly Ser Tyr Ser Ser
                645                 650                 655

Trp Ser Arg Val Met Val Asp Ser Lys Ala Thr Leu Gly Leu Gly Gly
            660                 665                 670

Val Ala Val Val Leu Gly Ala Val Met Ala Ala Met Gly Phe Phe Ser
        675                 680                 685

Tyr Leu Gly Ile Arg Ser Ser Leu Val Ile Leu Gln Val Val Pro Phe
    690                 695                 700

Leu Val Leu Ser Val Gly Ala Asp Asn Ile Phe Ile Phe Val Leu Glu
705                 710                 715                 720

Tyr Gln Arg Leu Pro Arg Arg Pro Gly Glu Pro Arg Glu Val His Ile
                725                 730                 735

Gly Arg Ala Leu Gly Arg Val Ala Pro Ser Met Leu Leu Cys Ser Leu
            740                 745                 750
```

```
Ser Glu Ala Ile Cys Phe Phe Leu Gly Ala Leu Thr Pro Met Pro Ala
        755                 760                 765

Val Arg Thr Phe Ala Leu Thr Ser Gly Leu Ala Val Ile Leu Asp Phe
    770                 775                 780

Leu Leu Gln Met Ser Ala Phe Val Ala Leu Leu Ser Leu Asp Ser Lys
785                 790                 795                 800

Arg Gln Glu Ala Ser Arg Leu Asp Val Cys Cys Cys Val Lys Pro Gln
                805                 810                 815

Glu Leu Pro Pro Pro Gly Gln Gly Glu Gly Leu Leu Leu Gly Phe Phe
            820                 825                 830

Gln Lys Ala Tyr Ala Pro Phe Leu Leu His Trp Ile Thr Arg Gly Val
        835                 840                 845

Val Leu Leu Leu Phe Leu Ala Leu Phe Gly Val Ser Leu Tyr Ser Met
    850                 855                 860

Cys His Ile Ser Val Gly Leu Asp Gln Glu Leu Ala Leu Pro Lys Asp
865                 870                 875                 880

Ser Tyr Leu Leu Asp Tyr Phe Leu Phe Leu Asn Arg Tyr Phe Glu Val
                885                 890                 895

Gly Ala Pro Val Tyr Phe Val Thr Thr Leu Gly Tyr Asn Phe Ser Ser
            900                 905                 910

Glu Ala Gly Met Asn Ala Ile Cys Ser Ser Ala Gly Cys Asn Asn Phe
        915                 920                 925

Ser Phe Thr Gln Lys Ile Gln Tyr Ala Thr Glu Phe Pro Glu Gln Ser
    930                 935                 940

Tyr Leu Ala Ile Pro Ala Ser Ser Trp Val Asp Asp Phe Ile Asp Trp
945                 950                 955                 960

Leu Thr Pro Ser Ser Cys Cys Arg Leu Tyr Ile Ser Gly Pro Asn Lys
                965                 970                 975

Asp Lys Phe Cys Pro Ser Thr Val Asn Ser Leu Asn Cys Leu Lys Asn
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980 985 990

Cys Met Ser Ile Thr Met Gly Ser Val Arg Pro Ser Val Glu Gln Phe
995 1000 1005

His Lys Tyr Leu Pro Trp Phe Leu Asn Asp Arg Pro Asn Ile Lys
1010 1015 1020

Cys Pro Lys Gly Gly Leu Ala Ala Tyr Ser Thr Ser Val Asn Leu
1025 1030 1035

Thr Ser Asp Gly Gln Val Leu Ala Ser Arg Phe Met Ala Tyr His
1040 1045 1050

Lys Pro Leu Lys Asn Ser Gln Asp Tyr Thr Glu Ala Leu Arg Ala
1055 1060 1065

Ala Arg Glu Leu Ala Ala Asn Ile Thr Ala Asp Leu Arg Lys Val
1070 1075 1080

Pro Gly Thr Asp Pro Ala Phe Glu Val Phe Pro Tyr Thr Ile Thr
1085 1090 1095

Asn Val Phe Tyr Glu Gln Tyr Leu Thr Ile Leu Pro Glu Gly Leu
1100 1105 1110

Phe Met Leu Ser Leu Cys Leu Val Pro Thr Phe Ala Val Ser Cys
1115 1120 1125

Leu Leu Leu Gly Leu Asp Leu Arg Ser Gly Leu Leu Asn Leu Leu
1130 1135 1140

Ser Ile Val Met Ile Leu Val Asp Thr Val Gly Phe Met Ala Leu
1145 1150 1155

Trp Asp Ile Ser Tyr Asn Ala Val Ser Leu Ile Asn Leu Val Ser
1160 1165 1170

Ala Val Gly Met Ser Val Glu Phe Val Ser His Ile Thr Arg Ser
1175 1180 1185

Phe Ala Ile Ser Thr Lys Pro Thr Trp Leu Glu Arg Ala Lys Glu
1190 1195 1200

Ala Thr Ile Ser Met Gly Ser Ala Val Phe Ala Gly Val Ala Met
1205 1210 1215

Thr Asn Leu Pro Gly Ile Leu Val Leu Gly Leu Ala Lys Ala Gln
1220 1225 1230

Leu Ile Gln Ile Phe Phe Phe Arg Leu Asn Leu Leu Ile Thr Leu
1235 1240 1245

Leu Gly Leu Leu His Gly Leu Val Phe Leu Pro Val Ile Leu Ser
1250 1255 1260

Tyr Val Gly Pro Asp Val Asn Pro Ala Leu Ala Leu Glu Gln Lys
1265 1270 1275

Arg Ala Glu Glu Ala Val Ala Ala Val Met Val Ala Ser Cys Pro
1280 1285 1290

Asn His Pro Ser Arg Val Ser Thr Ala Asp Asn Ile Tyr Val Asn
1295 1300 1305

His Ser Phe Glu Gly Ser Ile Lys Gly Ala Gly Ala Ile Ser Asn
1310 1315 1320

Phe Leu Pro Asn Asn Gly Arg Gln Phe
1325 1330

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<211> 885

<212> DNA

<213> Rattus sp.

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gcctccttca gtacttccag aacaaccgca ccctcctgat gctcacggcc aaccagactc 180

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cgctgatcat aaccttctct ctcaataact accccgctga tgatccccgc atggcccagg 420

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gcctaggtgg ggtgatagtg tgctgggagc agttctggct tgcatggggc ttctaactcc 720

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ctgtgggagc tggacacatc tacatcctag acttgagtac cagaggtacc taggaagccg 840

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<211> 458

<212> DNA

<213> Rattus sp.

<400> 6

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acggctttgc cccagaactt gcccacggag ctaatgctgc tagaagctct ttgcccaaaa 180

gtgaccaaaa gttctaatgg agtaggagct tgtccatgct tctgctgatg agggatcatg 240

aaggtcttcc ctctggttgt cctcaaggcc tgggggggagg ttgttcagag aaaaatggct 300

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gggatgcatc gctgttcatt aaaaaggcta tggctatg 458

<210> 7

<211> 896

<212> DNA

<213> Rattus sp.

<400> 7

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aggttatagc ctcccagttc atggcctacc acaagccctt aaggaactca caggacttca 180

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<210> 8

<211> 3124

<212> DNA

<213> Rattus sp.

<400> 8

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tccagaacaa ccgcaccctc ctgatgctca cggccaacca gactctgaat ggccagacct 180

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aaaa 3124

<210> 9

<211> 4484

<212> DNA

<213> Rattus sp.

<400> 9

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1               5                   10                  15

tcg gcc cag ggt gag ctc tac aca ccc act cac aaa gct ggc ttc tgc       96
Ser Ala Gln Gly Glu Leu Tyr Thr Pro Thr His Lys Ala Gly Phe Cys
            20                  25                  30

acc ttt tat gaa gag tgt ggg aag aac cca gag ctt tct gga ggc ctc      144
Thr Phe Tyr Glu Glu Cys Gly Lys Asn Pro Glu Leu Ser Gly Gly Leu
        35                  40                  45

aca tca cta tcc aat atc tcc tgc ttg tct aat acc cca gcc cgc cat      192
Thr Ser Leu Ser Asn Ile Ser Cys Leu Ser Asn Thr Pro Ala Arg His
    50                  55                  60

gtc aca ggt gac cac ctg gct ctt ctc cag cgc gtc tgt ccc cgc cta      240
Val Thr Gly Asp His Leu Ala Leu Leu Gln Arg Val Cys Pro Arg Leu
65                  70                  75                  80

tac aat ggc ccc aat gac acc tat gcc tgt tgc tct acc aag cag ctg      288
Tyr Asn Gly Pro Asn Asp Thr Tyr Ala Cys Cys Ser Thr Lys Gln Leu
                85                  90                  95

gtg tca tta gac agt agc ctg tct atc acc aag gcc ctc ctt aca cgc      336
Val Ser Leu Asp Ser Ser Leu Ser Ile Thr Lys Ala Leu Leu Thr Arg
            100                 105                 110

tgc ccg gca tgc tct gaa aat ttt gtg agc ata cac tgt cat aat acc      384
Cys Pro Ala Cys Ser Glu Asn Phe Val Ser Ile His Cys His Asn Thr
        115                 120                 125

tgc agc cct gac cag agc ctc ttc atc aat gtt act cgc gtg gtt cag      432
Cys Ser Pro Asp Gln Ser Leu Phe Ile Asn Val Thr Arg Val Val Gln
    130                 135                 140

cgg gac cct gga cag ctt cct gct gtg gtg gcc tat gag gcc ttt tat      480
Arg Asp Pro Gly Gln Leu Pro Ala Val Val Ala Tyr Glu Ala Phe Tyr
145                 150                 155                 160

caa cgc agt ttt gca gag aag gcc tat gag tcc tgt agc cgg gtg cgc      528
Gln Arg Ser Phe Ala Glu Lys Ala Tyr Glu Ser Cys Ser Arg Val Arg
                165                 170                 175

atc cct gca gct gcc tcg ctg gct gtg ggc agc atg tgt gga gtg tat      576
Ile Pro Ala Ala Ala Ser Leu Ala Val Gly Ser Met Cys Gly Val Tyr
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Gly Ser Ala Leu Cys Asn Ala Gln Arg Trp Leu Asn Phe Gln Gly Asp
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aca ggg aat ggc ctg gct ccg ctg gac atc acc ttc cac ctc ttg gag      672
Thr Gly Asn Gly Leu Ala Pro Leu Asp Ile Thr Phe His Leu Leu Glu
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cct ggc cag gcc ctg gca gat ggg atg aag cca ctg gat ggg aag atc      720
Pro Gly Gln Ala Leu Ala Asp Gly Met Lys Pro Leu Asp Gly Lys Ile
225                 230                 235                 240

aca ccc tgc aat gag tcc cag ggt gaa gac tcg gca gcc tgt tcc tgc      768
Thr Pro Cys Asn Glu Ser Gln Gly Glu Asp Ser Ala Ala Cys Ser Cys
                245                 250                 255

cag gac tgt gca gca tcc tgc cct gtc atc cct ccg ccc ccg gcc ctg      816
Gln Asp Cys Ala Ala Ser Cys Pro Val Ile Pro Pro Pro Pro Ala Leu
            260                 265                 270

cgc cct tct ttc tac atg ggt cga atg cca ggc tgg ctg gct ctc atc      864
Arg Pro Ser Phe Tyr Met Gly Arg Met Pro Gly Trp Leu Ala Leu Ile
        275                 280                 285

atc atc ttc act gct gtc ttt gta ttg ctc tct gtt gtc ctt gtg tat      912
Ile Ile Phe Thr Ala Val Phe Val Leu Leu Ser Val Val Leu Val Tyr
    290                 295                 300

ctc cga gtg gct tcc aac agg aac aag aac aag aca gca ggc tcc cag      960
Leu Arg Val Ala Ser Asn Arg Asn Lys Asn Lys Thr Ala Gly Ser Gln
305                 310                 315                 320

gaa gcc ccc aac ctc cct cgt aag cgc aga ttc tca cct cac act gtc     1008
Glu Ala Pro Asn Leu Pro Arg Lys Arg Arg Phe Ser Pro His Thr Val
                325                 330                 335

ctt ggc cgg ttc ttc gag agc tgg gga aca agg gtg gcc tca tgg cca     1056
Leu Gly Arg Phe Phe Glu Ser Trp Gly Thr Arg Val Ala Ser Trp Pro
            340                 345                 350

ctc act gtc ttg gca ctg tcc ttc ata gtt gtg ata gcc ttg tca gta     1104
Leu Thr Val Leu Ala Leu Ser Phe Ile Val Val Ile Ala Leu Ser Val
        355                 360                 365

ggc ctg acc ttt ata gaa ctc acc aca gac cct gtg gaa ctg tgg tcg     1152
Gly Leu Thr Phe Ile Glu Leu Thr Thr Asp Pro Val Glu Leu Trp Ser
    370                 375                 380

gcc cct aaa agc caa gcc cgg aaa gaa aag gct ttc cat gac gag cat     1200
Ala Pro Lys Ser Gln Ala Arg Lys Glu Lys Ala Phe His Asp Glu His
385                 390                 395                 400

ttt ggc ccc ttc ttc cga acc aac cag att ttt gtg aca gct aag aac     1248
Phe Gly Pro Phe Phe Arg Thr Asn Gln Ile Phe Val Thr Ala Lys Asn
                405                 410                 415

agg tcc agc tac aag tac gac tcc ctg ctg cta ggg ccc aag aac ttc     1296
Arg Ser Ser Tyr Lys Tyr Asp Ser Leu Leu Leu Gly Pro Lys Asn Phe
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agt ggg atc cta tcc ctg gac ttg ctg cag gag ctg ttg gag cta cag     1344
Ser Gly Ile Leu Ser Leu Asp Leu Leu Gln Glu Leu Leu Glu Leu Gln
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gag aga ctt cga cac ctg caa gtg tgg tcc cat gag gca cag cgc aac     1392
Glu Arg Leu Arg His Leu Gln Val Trp Ser His Glu Ala Gln Arg Asn
    450                 455                 460

atc tcc ctc cag gac atc tgc tat gct ccc ctc aac ccg cat aac acc     1440
Ile Ser Leu Gln Asp Ile Cys Tyr Ala Pro Leu Asn Pro His Asn Thr
465                 470                 475                 480

agc ctc act gac tgc tgt gtc aac agc ctc ctt caa tac ttc cag aac     1488
Ser Leu Thr Asp Cys Cys Val Asn Ser Leu Leu Gln Tyr Phe Gln Asn
                485                 490                 495

aac cac aca ctc ctg ctg ctc aca gcc aat cag act ctg aat ggc cag     1536
Asn His Thr Leu Leu Leu Leu Thr Ala Asn Gln Thr Leu Asn Gly Gln
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acc tcc ctg gtg gac tgg aag gac cat ttc ctc tac tgt gcc aat gcc     1584
Thr Ser Leu Val Asp Trp Lys Asp His Phe Leu Tyr Cys Ala Asn Ala
        515                 520                 525

cct ctc acg tac aaa gat ggc aca gcc ctg gcc ctg agc tgc ata gct     1632
Pro Leu Thr Tyr Lys Asp Gly Thr Ala Leu Ala Leu Ser Cys Ile Ala
    530                 535                 540

gac tac ggg gca cct gtc ttc ccc ttc ctt gct gtt ggg ggc tac caa     1680
Asp Tyr Gly Ala Pro Val Phe Pro Phe Leu Ala Val Gly Gly Tyr Gln
545                 550                 555                 560

ggg acg gac tac tcg gag gca gaa gcc ctg atc ata acc ttc tct atc     1728
Gly Thr Asp Tyr Ser Glu Ala Glu Ala Leu Ile Ile Thr Phe Ser Ile
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aat aac tac ccc gct gat gat ccc cgc atg gcc cac gcc aag ctc tgg     1776
Asn Asn Tyr Pro Ala Asp Asp Pro Arg Met Ala His Ala Lys Leu Trp
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gag gag gct ttc ttg aag gaa atg caa tcc ttc cag aga agc aca gct     1824
Glu Glu Ala Phe Leu Lys Glu Met Gln Ser Phe Gln Arg Ser Thr Ala
        595                 600                 605

gac aag ttc cag att gcg ttc tca gct gag cgt tct ctg gag gac gag     1872
Asp Lys Phe Gln Ile Ala Phe Ser Ala Glu Arg Ser Leu Glu Asp Glu
    610                 615                 620

atc aat cgc act acc atc cag gac ctg cct gtc ttt gcc atc agc tac     1920
Ile Asn Arg Thr Thr Ile Gln Asp Leu Pro Val Phe Ala Ile Ser Tyr
625                 630                 635                 640

ctt atc gtc ttc ctg tac atc tcc ctg gcc ctg ggc agc tac tcc aga     1968
Leu Ile Val Phe Leu Tyr Ile Ser Leu Ala Leu Gly Ser Tyr Ser Arg
                645                 650                 655

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Trp Ser Arg Val Ala Val Asp Ser Lys Ala Thr Leu Gly Leu Gly Gly
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Val Ala Val Val Leu Gly Ala Val Val Ala Ala Met Gly Phe Tyr Ser
        675                 680                 685

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Tyr Leu Gly Val Pro Ser Ser Leu Val Ile Ile Gln Val Val Pro Phe
    690                 695                 700

ctg gtg ctg gct gtg gga gct gac aac atc ttc atc ttt gtt ctt gag     2160
Leu Val Leu Ala Val Gly Ala Asp Asn Ile Phe Ile Phe Val Leu Glu
705                 710                 715                 720

tac cag agg ctg cct agg atg ccc ggg gag cag cga gag gct cac att     2208
Tyr Gln Arg Leu Pro Arg Met Pro Gly Glu Gln Arg Glu Ala His Ile
                725                 730                 735

ggc cgc acc ctg ggt agt gtg gcc ccc agc atg ctg ctg tgc agc ctc     2256
Gly Arg Thr Leu Gly Ser Val Ala Pro Ser Met Leu Leu Cys Ser Leu
            740                 745                 750

tct gag gcc atc tgc ttc ttt cta ggg gcc ctg acc tcc atg cca gct     2304
Ser Glu Ala Ile Cys Phe Phe Leu Gly Ala Leu Thr Ser Met Pro Ala
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gtg agg acc ttt gcc ttg acc tct ggc tta gca atc atc ttt gac ttc     2352
Val Arg Thr Phe Ala Leu Thr Ser Gly Leu Ala Ile Ile Phe Asp Phe
    770                 775                 780

ctg ctc cag atg aca gcc ttt gtg gcc ctg ctc tcc ctg gat agc aag     2400
Leu Leu Gln Met Thr Ala Phe Val Ala Leu Leu Ser Leu Asp Ser Lys
785                 790                 795                 800

agg cag gag gcc tct cgc ccc gac gtc gtg tgc tgc ttt tca agc cga     2448
Arg Gln Glu Ala Ser Arg Pro Asp Val Val Cys Cys Phe Ser Ser Arg
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aat ctg ccc cca ccg aaa caa aaa gaa ggc ctc tta ctt tgc ttc ttc     2496
Asn Leu Pro Pro Pro Lys Gln Lys Glu Gly Leu Leu Leu Cys Phe Phe
            820                 825                 830

cgc aag ata tac act ccc ttc ctg ctg cac aga ttc atc cgc cct gtt     2544
Arg Lys Ile Tyr Thr Pro Phe Leu Leu His Arg Phe Ile Arg Pro Val
        835                 840                 845

gtg ctg ctg ctc ttt ctg gtc ctg ttt gga gca aac ctc tac tta atg     2592
Val Leu Leu Leu Phe Leu Val Leu Phe Gly Ala Asn Leu Tyr Leu Met
    850                 855                 860

tgc aac atc agc gtg ggg ctg gac cag gat ctg gct ctg ccc aag gat     2640
Cys Asn Ile Ser Val Gly Leu Asp Gln Asp Leu Ala Leu Pro Lys Asp
865                 870                 875                 880

tcc tac ctg ata gac tac ttc ctc ttt ctg aac cgg tac ttg gaa gtg     2688
Ser Tyr Leu Ile Asp Tyr Phe Leu Phe Leu Asn Arg Tyr Leu Glu Val
                885                 890                 895

ggg cct cca gtg tac ttt gac acc acc tca ggc tac aac ttt tcc acc     2736
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Gly Pro Pro Val Tyr Phe Asp Thr Thr Ser Gly Tyr Asn Phe Ser Thr
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gag gca ggc atg aac gcc att tgc tct agt gca ggc tgt gag agc ttc     2784
Glu Ala Gly Met Asn Ala Ile Cys Ser Ser Ala Gly Cys Glu Ser Phe
        915                 920                 925

tcc cta acc cag aaa atc cag tat gcc agt gaa ttc cct aat cag tct     2832
Ser Leu Thr Gln Lys Ile Gln Tyr Ala Ser Glu Phe Pro Asn Gln Ser
    930                 935                 940

tat gtg gct att gct gca tcc tcc tgg gta gat gac ttc atc gac tgg     2880
Tyr Val Ala Ile Ala Ala Ser Ser Trp Val Asp Asp Phe Ile Asp Trp
945                 950                 955                 960

ctg acc cca tcc tcc tcc tgc tgc cgc att tat acc cgt ggc ccc cat     2928
Leu Thr Pro Ser Ser Ser Cys Cys Arg Ile Tyr Thr Arg Gly Pro His
                965                 970                 975

aaa gat gag ttc tgt ccc tca acg gat act tcc ttc aac tgt ctc aaa     2976
Lys Asp Glu Phe Cys Pro Ser Thr Asp Thr Ser Phe Asn Cys Leu Lys
            980                 985                 990

aac tgc atg aac cgc act ctg ggt  ccc gtg aga ccc aca  aca gaa cag   3024
Asn Cys Met Asn Arg Thr Leu Gly  Pro Val Arg Pro Thr  Thr Glu Gln
        995                 1000                1005

ttt cat  aag tac ctg ccc tgg  ttc ctg aat gat acg  ccc aac atc      3069
Phe His  Lys Tyr Leu Pro Trp  Phe Leu Asn Asp Thr  Pro Asn Ile
    1010                1015                1020

aga tgt  cct aaa ggg ggc cta  gca gcg tat aga acc  tct gtg aat      3114
Arg Cys  Pro Lys Gly Gly Leu  Ala Ala Tyr Arg Thr  Ser Val Asn
    1025                1030                1035

ttg agc  tca gat ggc cag att  ata gcc tcc cag ttc  atg gcc tac      3159
Leu Ser  Ser Asp Gly Gln Ile  Ile Ala Ser Gln Phe  Met Ala Tyr
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cac aag  ccc tta cgg aac tca  cag gac ttt aca gaa  gct ctc cgg      3204
His Lys  Pro Leu Arg Asn Ser  Gln Asp Phe Thr Glu  Ala Leu Arg
    1055                1060                1065

gca tcc  cgg ttg cta gca gcc  aac atc aca gct gaa  cta cgg aag      3249
Ala Ser  Arg Leu Leu Ala Ala  Asn Ile Thr Ala Glu  Leu Arg Lys
    1070                1075                1080

gtg cct  ggg aca gat ccc aac  ttt gag gtc ttc cct  tac acg atc      3294
Val Pro  Gly Thr Asp Pro Asn  Phe Glu Val Phe Pro  Tyr Thr Ile
    1085                1090                1095

tcc aat  gtg ttc tac cag caa  tac ctg acg gtt ctc  cct gag gga      3339
Ser Asn  Val Phe Tyr Gln Gln  Tyr Leu Thr Val Leu  Pro Glu Gly
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atc ttc  act ctt gct ctc tgc  ttc gtg ccc acc ttt  gtg gtc tgc      3384
Ile Phe  Thr Leu Ala Leu Cys  Phe Val Pro Thr Phe  Val Val Cys
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tac ctc  cta ctg ggc ctg gac  ata cgc tca ggc atc  ctc aac ctg      3429
Tyr Leu  Leu Leu Gly Leu Asp  Ile Arg Ser Gly Ile  Leu Asn Leu
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ctc tcc  atc att atg atc ctc  gtg gac acc atc ggc  ctc atg gct      3474
Leu Ser  Ile Ile Met Ile Leu  Val Asp Thr Ile Gly  Leu Met Ala
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gtg tgg  ggt atc agc tac aat  gct gtg tcc ctc atc  aac ctt gtc      3519
Val Trp  Gly Ile Ser Tyr Asn  Ala Val Ser Leu Ile  Asn Leu Val
    1160                 1165                 1170

acg gca  gtg ggc atg tct gtg  gag ttc gtg tcc cac  att acc cgg      3564
Thr Ala  Val Gly Met Ser Val  Glu Phe Val Ser His  Ile Thr Arg
    1175                 1180                 1185

tcc ttt  gct gta agc acc aag  cct acc cgg ctg gag  aga gcc aaa      3609
Ser Phe  Ala Val Ser Thr Lys  Pro Thr Arg Leu Glu  Arg Ala Lys
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gat gct  act atc ttc atg ggc  agt gcg gtg ttt gct  gga gtg gcc      3654
Asp Ala  Thr Ile Phe Met Gly  Ser Ala Val Phe Ala  Gly Val Ala
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atg acc  aac ttc ccg ggc atc  ctc atc ctg ggc ttt  gct cag gcc      3699
Met Thr  Asn Phe Pro Gly Ile  Leu Ile Leu Gly Phe  Ala Gln Ala
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cag ctt  atc cag att ttc ttc  ttc cgc ctc aac ctc  ctg atc acc      3744
Gln Leu  Ile Gln Ile Phe Phe  Phe Arg Leu Asn Leu  Leu Ile Thr
    1235                 1240                 1245

ttg ctg  ggt ctg cta cac ggc  ctg gtc ttc ctg ccc  gtt gtc ctc      3789
Leu Leu  Gly Leu Leu His Gly  Leu Val Phe Leu Pro  Val Val Leu
    1250                 1255                 1260

agc tat  ctg ggg cca gat gtt  aac caa gct ctg gta  ctg gag gag      3834
Ser Tyr  Leu Gly Pro Asp Val  Asn Gln Ala Leu Val  Leu Glu Glu
    1265                 1270                 1275

aaa cta  gcc act gag gca gcc  atg gtc tca gag cct  tct tgc cca      3879
Lys Leu  Ala Thr Glu Ala Ala  Met Val Ser Glu Pro  Ser Cys Pro
    1280                 1285                 1290

cag tac  ccc ttc ccg gct gat  gca aac acc agt gac  tat gtt aac      3924
Gln Tyr  Pro Phe Pro Ala Asp  Ala Asn Thr Ser Asp  Tyr Val Asn
    1295                 1300                 1305

tac ggc  ttt aat cca gaa ttt  atc cct gaa att aat  gct gct agc      3969
Tyr Gly  Phe Asn Pro Glu Phe  Ile Pro Glu Ile Asn  Ala Ala Ser
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Ser Ser  Leu Pro Lys Ser Asp  Gln Lys Phe
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Thr Phe Tyr Glu Glu Cys Gly Lys Asn Pro Glu Leu Ser Gly Gly Leu
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Thr Ser Leu Ser Asn Ile Ser Cys Leu Ser Asn Thr Pro Ala Arg His
    50                  55                  60

Val Thr Gly Asp His Leu Ala Leu Leu Gln Arg Val Cys Pro Arg Leu
65                  70                  75                  80

Tyr Asn Gly Pro Asn Asp Thr Tyr Ala Cys Cys Ser Thr Lys Gln Leu
                85                  90                  95

Val Ser Leu Asp Ser Ser Leu Ser Ile Thr Lys Ala Leu Leu Thr Arg
            100                 105                 110

Cys Pro Ala Cys Ser Glu Asn Phe Val Ser Ile His Cys His Asn Thr
        115                 120                 125

Cys Ser Pro Asp Gln Ser Leu Phe Ile Asn Val Thr Arg Val Val Gln
    130                 135                 140

Arg Asp Pro Gly Gln Leu Pro Ala Val Val Ala Tyr Glu Ala Phe Tyr
145                 150                 155                 160

Gln Arg Ser Phe Ala Glu Lys Ala Tyr Glu Ser Cys Ser Arg Val Arg
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Ile Pro Ala Ala Ala Ser Leu Ala Val Gly Ser Met Cys Gly Val Tyr
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Gly Ser Ala Leu Cys Asn Ala Gln Arg Trp Leu Asn Phe Gln Gly Asp
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Thr Gly Asn Gly Leu Ala Pro Leu Asp Ile Thr Phe His Leu Leu Glu
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Pro Gly Gln Ala Leu Ala Asp Gly Met Lys Pro Leu Asp Gly Lys Ile
225                 230                 235                 240

Thr Pro Cys Asn Glu Ser Gln Gly Glu Asp Ser Ala Ala Cys Ser Cys
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Gln Asp Cys Ala Ala Ser Cys Pro Val Ile Pro Pro Pro Pro Ala Leu
            260                 265                 270

Arg Pro Ser Phe Tyr Met Gly Arg Met Pro Gly Trp Leu Ala Leu Ile
        275                 280                 285

Ile Ile Phe Thr Ala Val Phe Val Leu Leu Ser Val Val Leu Val Tyr
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Leu Arg Val Ala Ser Asn Arg Asn Lys Asn Lys Thr Ala Gly Ser Gln
305                 310                 315                 320

Glu Ala Pro Asn Leu Pro Arg Lys Arg Arg Phe Ser Pro His Thr Val
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Leu Gly Arg Phe Phe Glu Ser Trp Gly Thr Arg Val Ala Ser Trp Pro
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Leu Thr Val Leu Ala Leu Ser Phe Ile Val Val Ile Ala Leu Ser Val
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Gly Leu Thr Phe Ile Glu Leu Thr Thr Asp Pro Val Glu Leu Trp Ser
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Ala Pro Lys Ser Gln Ala Arg Lys Glu Lys Ala Phe His Asp Glu His
385                 390                 395                 400

Phe Gly Pro Phe Phe Arg Thr Asn Gln Ile Phe Val Thr Ala Lys Asn
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Arg Ser Ser Tyr Lys Tyr Asp Ser Leu Leu Leu Gly Pro Lys Asn Phe
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Glu Arg Leu Arg His Leu Gln Val Trp Ser His Glu Ala Gln Arg Asn
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Ile Ser Leu Gln Asp Ile Cys Tyr Ala Pro Leu Asn Pro His Asn Thr
465 470 475 480

Ser Leu Thr Asp Cys Cys Val Asn Ser Leu Leu Gln Tyr Phe Gln Asn
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Asn His Thr Leu Leu Leu Leu Thr Ala Asn Gln Thr Leu Asn Gly Gln
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Thr Ser Leu Val Asp Trp Lys Asp His Phe Leu Tyr Cys Ala Asn Ala
515 520 525

Pro Leu Thr Tyr Lys Asp Gly Thr Ala Leu Ala Leu Ser Cys Ile Ala
530 535 540

Asp Tyr Gly Ala Pro Val Phe Pro Phe Leu Ala Val Gly Gly Tyr Gln
545 550 555 560

Gly Thr Asp Tyr Ser Glu Ala Glu Ala Leu Ile Ile Thr Phe Ser Ile
565 570 575

Asn Asn Tyr Pro Ala Asp Asp Pro Arg Met Ala His Ala Lys Leu Trp
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Glu Glu Ala Phe Leu Lys Glu Met Gln Ser Phe Gln Arg Ser Thr Ala
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Asp Lys Phe Gln Ile Ala Phe Ser Ala Glu Arg Ser Leu Glu Asp Glu
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Ile Asn Arg Thr Thr Ile Gln Asp Leu Pro Val Phe Ala Ile Ser Tyr
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Leu Ile Val Phe Leu Tyr Ile Ser Leu Ala Leu Gly Ser Tyr Ser Arg
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Trp Ser Arg Val Ala Val Asp Ser Lys Ala Thr Leu Gly Leu Gly Gly
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Val Ala Val Val Leu Gly Ala Val Val Ala Ala Met Gly Phe Tyr Ser
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Tyr Leu Gly Val Pro Ser Ser Leu Val Ile Ile Gln Val Val Pro Phe
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Leu Val Leu Ala Val Gly Ala Asp Asn Ile Phe Ile Phe Val Leu Glu
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Tyr Gln Arg Leu Pro Arg Met Pro Gly Glu Gln Arg Glu Ala His Ile
725 730 735

Gly Arg Thr Leu Gly Ser Val Ala Pro Ser Met Leu Leu Cys Ser Leu
740 745 750

Ser Glu Ala Ile Cys Phe Phe Leu Gly Ala Leu Thr Ser Met Pro Ala
755 760 765

Val Arg Thr Phe Ala Leu Thr Ser Gly Leu Ala Ile Ile Phe Asp Phe
770 775 780

Leu Leu Gln Met Thr Ala Phe Val Ala Leu Leu Ser Leu Asp Ser Lys
785 790 795 800

Arg Gln Glu Ala Ser Arg Pro Asp Val Val Cys Cys Phe Ser Ser Arg
805 810 815

Asn Leu Pro Pro Pro Lys Gln Lys Glu Gly Leu Leu Leu Cys Phe Phe
820 825 830

Arg Lys Ile Tyr Thr Pro Phe Leu Leu His Arg Phe Ile Arg Pro Val
835 840 845

Val Leu Leu Leu Phe Leu Val Leu Phe Gly Ala Asn Leu Tyr Leu Met
850 855 860

Cys Asn Ile Ser Val Gly Leu Asp Gln Asp Leu Ala Leu Pro Lys Asp
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Ser Tyr Leu Ile Asp Tyr Phe Leu Phe Leu Asn Arg Tyr Leu Glu Val
885 890 895

Gly Pro Pro Val Tyr Phe Asp Thr Thr Ser Gly Tyr Asn Phe Ser Thr
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Glu Ala Gly Met Asn Ala Ile Cys Ser Ser Ala Gly Cys Glu Ser Phe
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Ser Leu Thr Gln Lys Ile Gln Tyr Ala Ser Glu Phe Pro Asn Gln Ser
930 935 940

Tyr Val Ala Ile Ala Ala Ser Ser Trp Val Asp Asp Phe Ile Asp Trp
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Leu Thr Pro Ser Ser Ser Cys Cys Arg Ile Tyr Thr Arg Gly Pro His
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Lys Asp Glu Phe Cys Pro Ser Thr Asp Thr Ser Phe Asn Cys Leu Lys
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Asn Cys Met Asn Arg Thr Leu Gly Pro Val Arg Pro Thr Thr Glu Gln
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Phe His Lys Tyr Leu Pro Trp Phe Leu Asn Asp Thr Pro Asn Ile
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Leu Ser Ser Asp Gly Gln Ile Ile Ala Ser Gln Phe Met Ala Tyr
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Val Pro Gly Thr Asp Pro Asn Phe Glu Val Phe Pro Tyr Thr Ile
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Ile Phe Thr Leu Ala Leu Cys Phe Val Pro Thr Phe Val Val Cys
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Leu Leu Gly Leu Leu His Gly Leu Val Phe Leu Pro Val Val Leu
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Ser Tyr Leu Gly Pro Asp Val Asn Gln Ala Leu Val Leu Glu Glu
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Lys Leu Ala Thr Glu Ala Ala Met Val Ser Glu Pro Ser Cys Pro
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Gln Tyr Pro Phe Pro Ala Asp Ala Asn Thr Ser Asp Tyr Val Asn
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Ala Glu Ala Gly Leu Arg Gly Trp Leu Leu Trp Ala Leu Leu Leu Arg
            5                   10                  15

ttg gcc cag agt gag cct tac aca acc atc cac cag cct ggc tac tgc      155
Leu Ala Gln Ser Glu Pro Tyr Thr Thr Ile His Gln Pro Gly Tyr Cys
        20                  25                  30

gcc ttc tat gac gaa tgt ggg aag aac cca gag ctg tct gga agc ctc      203
Ala Phe Tyr Asp Glu Cys Gly Lys Asn Pro Glu Leu Ser Gly Ser Leu
    35                  40                  45

atg aca ctc tcc aac gtg tcc tgc ctg tcc aac acg ccg gcc cgc aag      251
Met Thr Leu Ser Asn Val Ser Cys Leu Ser Asn Thr Pro Ala Arg Lys
50                  55                  60                  65

atc aca ggt gat cac ctg atc cta tta cag aag atc tgc ccc cgc ctc      299
Ile Thr Gly Asp His Leu Ile Leu Leu Gln Lys Ile Cys Pro Arg Leu
                70                  75                  80

tac acc ggc ccc aac acc caa gcc tgc tgc tcc gcc aag cag ctg gta      347
Tyr Thr Gly Pro Asn Thr Gln Ala Cys Cys Ser Ala Lys Gln Leu Val
            85                  90                  95

tca ctg gaa gcg agt ctg tcg atc acc aag gcc ctc ctc acc cgc tgc      395
Ser Leu Glu Ala Ser Leu Ser Ile Thr Lys Ala Leu Leu Thr Arg Cys
        100                 105                 110

cca gcc tgc tct gac aat ttt gtg aac ctg cac tgc cac aac acg tgc      443
Pro Ala Cys Ser Asp Asn Phe Val Asn Leu His Cys His Asn Thr Cys
    115                 120                 125

agc ccc aat cag agc ctc ttc atc aat gtg acc cgc gtg gcc cag cta      491
Ser Pro Asn Gln Ser Leu Phe Ile Asn Val Thr Arg Val Ala Gln Leu
130                 135                 140                 145

ggg gct gga caa ctc cca gct gtg gtg gcc tat gag gcc ttc tac cag      539
Gly Ala Gly Gln Leu Pro Ala Val Val Ala Tyr Glu Ala Phe Tyr Gln
                150                 155                 160

cat agc ttt gcc gag cag agc tat gac tcc tgc agc cgt gtg cgc gtc      587
His Ser Phe Ala Glu Gln Ser Tyr Asp Ser Cys Ser Arg Val Arg Val
            165                 170                 175

cct gca gct gcc acg ctg gct gtg ggc acc atg tgt ggc gtg tat ggc      635
Pro Ala Ala Ala Thr Leu Ala Val Gly Thr Met Cys Gly Val Tyr Gly
        180                 185                 190

tct gcc ctt tgc aat gcc cag cgc tgg ctc aac ttc cag gga gac aca      683
Ser Ala Leu Cys Asn Ala Gln Arg Trp Leu Asn Phe Gln Gly Asp Thr
    195                 200                 205

ggc aat ggt ctg gcc cca ctg gac atc acc ttc cac ctc ttg gag cct      731
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Gly Asn Gly Leu Ala Pro Leu Asp Ile Thr Phe His Leu Leu Glu Pro
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ggc cag gcc gtg ggg agt ggg att cag cct ctg aat gag ggg gtt gca      779
Gly Gln Ala Val Gly Ser Gly Ile Gln Pro Leu Asn Glu Gly Val Ala
                230                 235                 240

cgt tgc aat gag tcc caa ggt gac gac gtg gcg acc tgc tcc tgc caa      827
Arg Cys Asn Glu Ser Gln Gly Asp Asp Val Ala Thr Cys Ser Cys Gln
            245                 250                 255

gac tgt gct gca tcc tgt cct gcc ata gcc cgc ccc cag gcc ctc gac      875
Asp Cys Ala Ala Ser Cys Pro Ala Ile Ala Arg Pro Gln Ala Leu Asp
        260                 265                 270

tcc acc ttc tac ctg ggc cag atg ccg ggc agt ctg gtc ctc atc atc      923
Ser Thr Phe Tyr Leu Gly Gln Met Pro Gly Ser Leu Val Leu Ile Ile
    275                 280                 285

atc ctc tgc tct gtc ttc gct gtg gtc acc atc ctg ctt gtg gga ttc      971
Ile Leu Cys Ser Val Phe Ala Val Val Thr Ile Leu Leu Val Gly Phe
290                 295                 300                 305

cgt gtg gcc ccc gcc agg gac aaa agc aag atg gtg gac ccc aag aag     1019
Arg Val Ala Pro Ala Arg Asp Lys Ser Lys Met Val Asp Pro Lys Lys
                310                 315                 320

ggc acc agc ctc tct gac aag ctc agc ttc tcc acc cac acc ctc ctt     1067
Gly Thr Ser Leu Ser Asp Lys Leu Ser Phe Ser Thr His Thr Leu Leu
            325                 330                 335

ggc cag ttc ttc cag ggc tgg ggc acg tgg gtg gct tcg tgg cct ctg     1115
Gly Gln Phe Phe Gln Gly Trp Gly Thr Trp Val Ala Ser Trp Pro Leu
        340                 345                 350

acc atc ttg gtg cta tct gtc atc ccg gtg gtg gcc ttg gca gcg ggc     1163
Thr Ile Leu Val Leu Ser Val Ile Pro Val Val Ala Leu Ala Ala Gly
    355                 360                 365

ctg gtc ttt aca gaa ctc act acg gac ccc gtg gag ctg tgg tcg gcc     1211
Leu Val Phe Thr Glu Leu Thr Thr Asp Pro Val Glu Leu Trp Ser Ala
370                 375                 380                 385

ccc aac agc caa gcc cgg agt gag aaa gct ttc cat gac cag cat ttc     1259
Pro Asn Ser Gln Ala Arg Ser Glu Lys Ala Phe His Asp Gln His Phe
                390                 395                 400

ggc ccc ttc ttc cga acc aac cag gtg atc ctg acg gct cct aac cgg     1307
Gly Pro Phe Phe Arg Thr Asn Gln Val Ile Leu Thr Ala Pro Asn Arg
            405                 410                 415

tcc agc tac agg tat gac tct ctg ctg ctg ggg ccc aag aac ttc agc     1355
Ser Ser Tyr Arg Tyr Asp Ser Leu Leu Leu Gly Pro Lys Asn Phe Ser
        420                 425                 430

gga atc ctg gac ctg gac ttg ctg ctg gag ctg cta gag ctg cag gag     1403
Gly Ile Leu Asp Leu Asp Leu Leu Leu Glu Leu Leu Glu Leu Gln Glu
    435                 440                 445
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agg ctg cgg cac ctc cag gta tgg tcg ccc gaa gca cag cgc aac atc     1451
Arg Leu Arg His Leu Gln Val Trp Ser Pro Glu Ala Gln Arg Asn Ile
450                 455                 460                 465

tcc ctg cag gac atc tgc tac gcc ccc ctc aat ccg gac aat acc agt     1499
Ser Leu Gln Asp Ile Cys Tyr Ala Pro Leu Asn Pro Asp Asn Thr Ser
                470                 475                 480

ctc tac gac tgc tgc atc aac agc ctc ctg cag tat ttc cag aac aac     1547
Leu Tyr Asp Cys Cys Ile Asn Ser Leu Leu Gln Tyr Phe Gln Asn Asn
            485                 490                 495

cgc acg ctc ctg ctg ctc aca gcc aac cag aca ctg atg ggg cag acc     1595
Arg Thr Leu Leu Leu Leu Thr Ala Asn Gln Thr Leu Met Gly Gln Thr
        500                 505                 510

tcc caa gtc gac tgg aag gac cat ttt ctg tac tgt gcc aat gcc ccg     1643
Ser Gln Val Asp Trp Lys Asp His Phe Leu Tyr Cys Ala Asn Ala Pro
    515                 520                 525

ctc acc ttc aag gat ggc aca gcc ctg gcc ctg agc tgc atg gct gac     1691
Leu Thr Phe Lys Asp Gly Thr Ala Leu Ala Leu Ser Cys Met Ala Asp
530                 535                 540                 545

tac ggg gcc cct gtc ttc ccc ttc ctt gcc att ggg ggg tac aaa gga     1739
Tyr Gly Ala Pro Val Phe Pro Phe Leu Ala Ile Gly Gly Tyr Lys Gly
                550                 555                 560

aag gac tat tct gag gca gag gcc ctg atc atg acg ttc tcc ctc aac     1787
Lys Asp Tyr Ser Glu Ala Glu Ala Leu Ile Met Thr Phe Ser Leu Asn
            565                 570                 575

aat tac cct gcc ggg gac ccc cgt ctg gcc cag gcc aag ctg tgg gag     1835
Asn Tyr Pro Ala Gly Asp Pro Arg Leu Ala Gln Ala Lys Leu Trp Glu
        580                 585                 590

gag gcc ttc tta gag gaa atg cga gcc ttc cag cgt cgg atg gct ggc     1883
Glu Ala Phe Leu Glu Glu Met Arg Ala Phe Gln Arg Arg Met Ala Gly
    595                 600                 605

atg ttc cag gtc acg ttc atg gct gag cgc tct ctg gaa gac gag atc     1931
Met Phe Gln Val Thr Phe Met Ala Glu Arg Ser Leu Glu Asp Glu Ile
610                 615                 620                 625

aat cgc acc aca gct gaa gac ctg ccc atc ttt gcc acc agc tac att     1979
Asn Arg Thr Thr Ala Glu Asp Leu Pro Ile Phe Ala Thr Ser Tyr Ile
                630                 635                 640

gtc ata ttc ctg tac atc tct ctg gcc ctg ggc agc tat tcc agc tgg     2027
Val Ile Phe Leu Tyr Ile Ser Leu Ala Leu Gly Ser Tyr Ser Ser Trp
            645                 650                 655

agc cga gtg atg gtg gac tcc aag gcc acg ctg ggc ctc ggc ggg gtg     2075
Ser Arg Val Met Val Asp Ser Lys Ala Thr Leu Gly Leu Gly Gly Val
        660                 665                 670

gcc gtg gtc ctg gga gca gtc atg gct gcc atg ggc ttc ttc tcc tac     2123
Ala Val Val Leu Gly Ala Val Met Ala Ala Met Gly Phe Phe Ser Tyr
    675                 680                 685
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ttg ggt atc cgc tcc tcc ctg gtc atc ctg caa gtg gtt cct ttc ctg     2171
Leu Gly Ile Arg Ser Ser Leu Val Ile Leu Gln Val Val Pro Phe Leu
690                 695                 700                 705

gtg ctg tcc gtg ggg gct gat aac atc ttc atc ttt gtt ctc gag tac     2219
Val Leu Ser Val Gly Ala Asp Asn Ile Phe Ile Phe Val Leu Glu Tyr
                710                 715                 720

cag agg ctg ccc cgg agg cct ggg gag cca cga gag gtc cac att ggg     2267
Gln Arg Leu Pro Arg Arg Pro Gly Glu Pro Arg Glu Val His Ile Gly
            725                 730                 735

cga gcc cta ggc agg gtg gct ccc agc atg ctg ttg tgc agc ctc tct     2315
Arg Ala Leu Gly Arg Val Ala Pro Ser Met Leu Leu Cys Ser Leu Ser
        740                 745                 750

gag gcc atc tgc ttc ttc cta ggg gcc ctg acc ccc atg cca gct gtg     2363
Glu Ala Ile Cys Phe Phe Leu Gly Ala Leu Thr Pro Met Pro Ala Val
    755                 760                 765

cgg acc ttt gcc ctg acc tct ggc ctt gca gtg atc ctt gac ttc ctc     2411
Arg Thr Phe Ala Leu Thr Ser Gly Leu Ala Val Ile Leu Asp Phe Leu
770                 775                 780                 785

ctg cag atg tca gcc ttt gtg gcc ctg ctc tcc ctg gac agc aag agg     2459
Leu Gln Met Ser Ala Phe Val Ala Leu Leu Ser Leu Asp Ser Lys Arg
                790                 795                 800

cag gag gcc tcc cgg ttg gac gtc tgc tgc tgt gtc aag ccc cag gag     2507
Gln Glu Ala Ser Arg Leu Asp Val Cys Cys Cys Val Lys Pro Gln Glu
            805                 810                 815

ctg ccc ccg cct ggc cag gga gag ggg ctc ctg ctt ggc ttc ttc caa     2555
Leu Pro Pro Pro Gly Gln Gly Glu Gly Leu Leu Leu Gly Phe Phe Gln
        820                 825                 830

aag gct tat gcc ccc ttc ctg ctg cac tgg atc act cga ggt gtt gtg     2603
Lys Ala Tyr Ala Pro Phe Leu Leu His Trp Ile Thr Arg Gly Val Val
    835                 840                 845

ctg ctg ctg ttt ctc gcc ctg ttc gga gtg agc ctc tac tcc atg tgc     2651
Leu Leu Leu Phe Leu Ala Leu Phe Gly Val Ser Leu Tyr Ser Met Cys
850                 855                 860                 865

cac atc agc gtg gga ctg gac cag gag ctg gcc ctg ccc aag gac tcg     2699
His Ile Ser Val Gly Leu Asp Gln Glu Leu Ala Leu Pro Lys Asp Ser
                870                 875                 880

tac ctg ctt gac tat ttc ctc ttt ctg aac cgc tac ttc gag gtg ggg     2747
Tyr Leu Leu Asp Tyr Phe Leu Phe Leu Asn Arg Tyr Phe Glu Val Gly
            885                 890                 895

gcc ccg gtg tac ttt gtt acc acc ttg ggc tac aac ttc tcc agc gag     2795
Ala Pro Val Tyr Phe Val Thr Thr Leu Gly Tyr Asn Phe Ser Ser Glu
        900                 905                 910

gct ggg atg aat gcc atc tgc tcc agt gca ggc tgc aac aac ttc tcc     2843
Ala Gly Met Asn Ala Ile Cys Ser Ser Ala Gly Cys Asn Asn Phe Ser
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    915                 920                 925

ttc acc cag aag atc cag tat gcc aca gag ttc cct gag cag tct tac     2891
Phe Thr Gln Lys Ile Gln Tyr Ala Thr Glu Phe Pro Glu Gln Ser Tyr
930                 935                 940                 945

ctg gcc atc cct gcc tcc tcc tgg gtg gat gac ttc att gac tgg ctg     2939
Leu Ala Ile Pro Ala Ser Ser Trp Val Asp Asp Phe Ile Asp Trp Leu
                950                 955                 960

acc ccg tcc tcc tgc tgc cgc ctt tat ata tct ggc ccc aat aag gac     2987
Thr Pro Ser Ser Cys Cys Arg Leu Tyr Ile Ser Gly Pro Asn Lys Asp
            965                 970                 975

aag ttc tgc ccc tcg acc gtc aac tct ctg aac tgc cta aag aac tgc     3035
Lys Phe Cys Pro Ser Thr Val Asn Ser Leu Asn Cys Leu Lys Asn Cys
        980                 985                 990

atg agc atc acg atg ggc tct  gtg agg ccc tcg gtg  gag cag ttc cat   3083
Met Ser Ile Thr Met Gly Ser  Val Arg Pro Ser Val  Glu Gln Phe His
    995                 1000                1005

aag  tat ctt ccc tgg ttc  ctg aac gac cgg ccc  aac atc aaa tgt      3128
Lys  Tyr Leu Pro Trp Phe  Leu Asn Asp Arg Pro  Asn Ile Lys Cys
1010                1015                1020

ccc  aaa ggc ggc ctg gca  gca tac agc acc tct  gtg aac ttg act      3173
Pro  Lys Gly Gly Leu Ala  Ala Tyr Ser Thr Ser  Val Asn Leu Thr
1025                1030                1035

tca  gat ggc cag gtt tta  gac aca gtt gcc att  ctg tca ccc agg      3218
Ser  Asp Gly Gln Val Leu  Asp Thr Val Ala Ile  Leu Ser Pro Arg
1040                1045                1050

ctg  gag tac agt ggc aca  atc tcg gct cac tgc  aac ctc tac ctc      3263
Leu  Glu Tyr Ser Gly Thr  Ile Ser Ala His Cys  Asn Leu Tyr Leu
1055                1060                1065

ctg  gat tca gcc tcc agg  ttc atg gcc tat cac  aag ccc ctg aaa      3308
Leu  Asp Ser Ala Ser Arg  Phe Met Ala Tyr His  Lys Pro Leu Lys
1070                1075                1080

aac  tca cag gat tac aca  gaa gct ctg cgg gca  gct cga gag ctg      3353
Asn  Ser Gln Asp Tyr Thr  Glu Ala Leu Arg Ala  Ala Arg Glu Leu
1085                1090                1095

gca  gcc aac atc act gct  gac ctg cgg aaa gtg  cct gga aca gac      3398
Ala  Ala Asn Ile Thr Ala  Asp Leu Arg Lys Val  Pro Gly Thr Asp
1100                1105                1110

ccg  gct ttt gag gtc ttc  ccc tac acg atc acc  aat gtg ttt tat      3443
Pro  Ala Phe Glu Val Phe  Pro Tyr Thr Ile Thr  Asn Val Phe Tyr
1115                1120                1125

gag  cag tac ctg acc atc  ctc cct gag ggg ctc  ttc atg ctc agc      3488
Glu  Gln Tyr Leu Thr Ile  Leu Pro Glu Gly Leu  Phe Met Leu Ser
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ctc  tgc ctt gtg ccc acc  ttc gct gtc tcc tgc  ctc ctg ctg ggc      3533
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Leu  Asp Leu Arg Ser Gly  Leu Leu Asn Leu Leu  Ser Ile Val Met
1160                1165                1170

atc  ctc gtg gac act gtc  ggc ttc atg gcc ctg  tgg ggc atc agt      3623
Ile  Leu Val Asp Thr Val  Gly Phe Met Ala Leu  Trp Gly Ile Ser
1175                1180                1185

tac  aat gct gtg tcc ctc  atc aac ctg gtc tcg  gcg gtg ggc atg      3668
Tyr  Asn Ala Val Ser Leu  Ile Asn Leu Val Ser  Ala Val Gly Met
1190                1195                1200

tct  gtg gag ttt gtg tcc  cac att acc cgc tcc  ttt gcc atc agc      3713
Ser  Val Glu Phe Val Ser  His Ile Thr Arg Ser  Phe Ala Ile Ser
1205                1210                1215

acc  aag ccc acc tgg ctg  gag agg gcc aaa gag  gcc acc atc tct      3758
Thr  Lys Pro Thr Trp Leu  Glu Arg Ala Lys Glu  Ala Thr Ile Ser
1220                1225                1230

atg  gga agt gcg gtg ttt  gca ggt gtg gcc atg  acc aac ctg cct      3803
Met  Gly Ser Ala Val Phe  Ala Gly Val Ala Met  Thr Asn Leu Pro
1235                1240                1245

ggc  atc ctt gtc ctg ggc  ctc gcc aag gcc cag  ctc att cag atc      3848
Gly  Ile Leu Val Leu Gly  Leu Ala Lys Ala Gln  Leu Ile Gln Ile
1250                1255                1260

ttc  ttc ttc cgc ctc aac  ctc ctg atc act ctg  ctg ggc ctg ctg      3893
Phe  Phe Phe Arg Leu Asn  Leu Leu Ile Thr Leu  Leu Gly Leu Leu
1265                1270                1275

cat  ggc ttg gtc ttc ctg  ccc gtc atc ctc agc  tac gtg ggg cct      3938
His  Gly Leu Val Phe Leu  Pro Val Ile Leu Ser  Tyr Val Gly Pro
1280                1285                1290

gac  gtt aac ccg gct ctg  gca ctg gag cag aag  cgg gct gag gag      3983
Asp  Val Asn Pro Ala Leu  Ala Leu Glu Gln Lys  Arg Ala Glu Glu
1295                1300                1305

gcg  gtg gca gca gtc atg  gtg gcc tct tgc cca  aat cac ccc tcc      4028
Ala  Val Ala Ala Val Met  Val Ala Ser Cys Pro  Asn His Pro Ser
1310                1315                1320

cga  gtc tcc aca gct gac  aac atc tat gtc aac  cac agc ttt gaa      4073
Arg  Val Ser Thr Ala Asp  Asn Ile Tyr Val Asn  His Ser Phe Glu
1325                1330                1335

ggt  tct atc aaa ggt gct  ggt gcc atc agc aac  ttc ttg ccc aac      4118
Gly  Ser Ile Lys Gly Ala  Gly Ala Ile Ser Asn  Phe Leu Pro Asn
1340                1345                1350

aat  ggg cgg cag ttc tga tacagccaga ggccctgtct aggctctatg          4166
Asn  Gly Arg Gln Phe
1355
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gccctgaacc aaagggttat ggggatcttc cttgtgactg ccccttgaca cacgccctcc 4226

tcaaatccta ggggaggcca ttcccatgag actgcctgtc actggaggat ggcctgctct 4286

tgaggtatcc aggcagcacc actgatggct cctctgctcc catagtgggt ccccagtttc 4346

caagtcacct aggccttggg cagtgcctcc tcctgggcct gggtctggaa gttggcagga 4406

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gagcttttgg acagctctcc agaagcatga actgcagtgg agatgaagat cctggctctg 5006

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Leu Met Thr Leu Ser Asn Val Ser Cys Leu Ser Asn Thr Pro Ala Arg
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Lys Ile Thr Gly Asp His Leu Ile Leu Leu Gln Lys Ile Cys Pro Arg
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Leu Tyr Thr Gly Pro Asn Thr Gln Ala Cys Cys Ser Ala Lys Gln Leu
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Val Ser Leu Glu Ala Ser Leu Ser Ile Thr Lys Ala Leu Leu Thr Arg
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Cys Pro Ala Cys Ser Asp Asn Phe Val Asn Leu His Cys His Asn Thr
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Cys Ser Pro Asn Gln Ser Leu Phe Ile Asn Val Thr Arg Val Ala Gln
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Leu Gly Ala Gly Gln Leu Pro Ala Val Val Ala Tyr Glu Ala Phe Tyr
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Gln His Ser Phe Ala Glu Gln Ser Tyr Asp Ser Cys Ser Arg Val Arg
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Val Pro Ala Ala Ala Thr Leu Ala Val Gly Thr Met Cys Gly Val Tyr
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Gly Ser Ala Leu Cys Asn Ala Gln Arg Trp Leu Asn Phe Gln Gly Asp
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Thr Gly Asn Gly Leu Ala Pro Leu Asp Ile Thr Phe His Leu Leu Glu
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Pro Gly Gln Ala Val Gly Ser Gly Ile Gln Pro Leu Asn Glu Gly Val
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Ala Arg Cys Asn Glu Ser Gln Gly Asp Asp Val Ala Thr Cys Ser Cys
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Gln Asp Cys Ala Ala Ser Cys Pro Ala Ile Ala Arg Pro Gln Ala Leu
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Asp Ser Thr Phe Tyr Leu Gly Gln Met Pro Gly Ser Leu Val Leu Ile
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Ile Ile Leu Cys Ser Val Phe Ala Val Val Thr Ile Leu Leu Val Gly

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Phe Arg Val Ala Pro Ala Arg Asp Lys Ser Lys Met Val Asp Pro Lys
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Lys Gly Thr Ser Leu Ser Asp Lys Leu Ser Phe Ser Thr His Thr Leu
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Leu Gly Gln Phe Phe Gln Gly Trp Gly Thr Trp Val Ala Ser Trp Pro
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Gly Leu Val Phe Thr Glu Leu Thr Thr Asp Pro Val Glu Leu Trp Ser
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Phe Gly Pro Phe Phe Arg Thr Asn Gln Val Ile Leu Thr Ala Pro Asn
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Arg Ser Ser Tyr Arg Tyr Asp Ser Leu Leu Leu Gly Pro Lys Asn Phe
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Ser Gly Ile Leu Asp Leu Asp Leu Leu Leu Glu Leu Leu Glu Leu Gln
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Ser Leu Tyr Asp Cys Cys Ile Asn Ser Leu Leu Gln Tyr Phe Gln Asn
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Asn Arg Thr Leu Leu Leu Leu Thr Ala Asn Gln Thr Leu Met Gly Gln
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Thr Ser Gln Val Asp Trp Lys Asp His Phe Leu Tyr Cys Ala Asn Ala
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Asp Tyr Gly Ala Pro Val Phe Pro Phe Leu Ala Ile Gly Gly Tyr Lys
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Gly Lys Asp Tyr Ser Glu Ala Glu Ala Leu Ile Met Thr Phe Ser Leu
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Asn Asn Tyr Pro Ala Gly Asp Pro Arg Leu Ala Gln Ala Lys Leu Trp
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Glu Glu Ala Phe Leu Glu Glu Met Arg Ala Phe Gln Arg Arg Met Ala
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Gly Met Phe Gln Val Thr Phe Met Ala Glu Arg Ser Leu Glu Asp Glu
    610                 615                 620

Ile Asn Arg Thr Thr Ala Glu Asp Leu Pro Ile Phe Ala Thr Ser Tyr
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Trp Ser Arg Val Met Val Asp Ser Lys Ala Thr Leu Gly Leu Gly Gly
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Tyr Leu Gly Ile Arg Ser Ser Leu Val Ile Leu Gln Val Val Pro Phe
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Leu Val Leu Ser Val Gly Ala Asp Asn Ile Phe Ile Phe Val Leu Glu
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Tyr Gln Arg Leu Pro Arg Arg Pro Gly Glu Pro Arg Glu Val His Ile
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Gly Arg Ala Leu Gly Arg Val Ala Pro Ser Met Leu Leu Cys Ser Leu
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Ser Glu Ala Ile Cys Phe Phe Leu Gly Ala Leu Thr Pro Met Pro Ala
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Arg Gln Glu Ala Ser Arg Leu Asp Val Cys Cys Cys Val Lys Pro Gln
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Glu Leu Pro Pro Pro Gly Gln Gly Glu Gly Leu Leu Leu Gly Phe Phe
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Gln Lys Ala Tyr Ala Pro Phe Leu Leu His Trp Ile Thr Arg Gly Val
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Val Leu Leu Leu Phe Leu Ala Leu Phe Gly Val Ser Leu Tyr Ser Met
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Cys His Ile Ser Val Gly Leu Asp Gln Glu Leu Ala Leu Pro Lys Asp
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Ser Tyr Leu Leu Asp Tyr Phe Leu Phe Leu Asn Arg Tyr Phe Glu Val
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Gly Ala Pro Val Tyr Phe Val Thr Thr Leu Gly Tyr Asn Phe Ser Ser
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Glu Ala Gly Met Asn Ala Ile Cys Ser Ser Ala Gly Cys Asn Asn Phe
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Ser Phe Thr Gln Lys Ile Gln Tyr Ala Thr Glu Phe Pro Glu Gln Ser
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Leu Thr Pro Ser Ser Cys Cys Arg Leu Tyr Ile Ser Gly Pro Asn Lys
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Asp Lys Phe Cys Pro Ser Thr Val Asn Ser Leu Asn Cys Leu Lys Asn
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Cys Met Ser Ile Thr Met Gly Ser  Val Arg Pro Ser Val  Glu Gln Phe
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Leu His Gly Leu Val Phe Leu Pro Val Ile Leu Ser Tyr Val Gly
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